Molecular Epidemiology and Transmission patterns of *Mycobacterium tuberculosis* complex in Afar Pastoral Communities and their livestock in Ethiopia

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Dissertation for the degree of Philosophiae Doctor (PhD)

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March 2014

Series of dissertations submitted to the
Faculty of Medicine, University of Oslo
No. 1937

ISBN 978-82-8264-871-4

Cover: Inger Sandved Anfinsen.
Printed in Norway: AIT Oslo AS.

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DEDICATION

To my father, MAMO KASSA and my mother, BALCHITU DEBELLA

for their firm belief in Education.

ACKNOWLEDGEMENTS

This study was done under the collaborative project between Aklilu Lemma Institute of Pathobiology, Addis Ababa University (Ethiopia) and Department of Community Medicine, Institute of Health and Society, Faculty of Medicine, University of Oslo (Norway) and funded by the Norwegian Programme for Development, Research and Education (NUFU PRO-2007/10198). Hence, my sincere acknowledgement goes to all the institutes and individual who contributed from conception to the success of this project.

I would like to express my deepest gratitude to my principal supervisor, Dr. Fekadu Abebe (University of Oslo) for his extensive scientific guidance, valuable comments and discussion in all aspects of the study including the study design, data collection, write-up of scientific papers, editing the thesis and overall facilitation of my study and research activities in both Ethiopia and Norway. It has been an honor for me to work under your supervision. I would like also to extend my special acknowledgement to Dr. Fekadu's family for their warm family welcome and support during my stay in Oslo and I thank you for those special occasions you dedicate for us and I always feel home.

I would like to express my in-depth gratitude to my co- supervisor, Dr. Gobena Ameni (Aklilu Lemma Institute of Pathobiology, Addis Ababa University, Ethiopia) for his full dedication to support me to join this study, sharing his extensive scientific knowledge, friendly advice throughout my study. It gives me a great satisfaction to work with you and share our common scientific field of interest on tuberculosis. I would like also to extend my gratitude to Dr. Gobena's family for their support during my arrival and departure of my journey abroad.

My special thank goes to my co- supervisor, Professor Gunnar Aksel Bjune (Department of Community Medicine, Institute of Health and Society, University of Oslo, Norway) for his extensive scientific guidance, valuable comments and support during the study design, data collection, scientific discussion and write-up of the papers and the thesis. I am very grateful for your critical support at the final part of my work in arranging laboratory facility and training on the molecular techniques at The Norwegian Institute for Public health (FHI) in Oslo, Norway.

I would like to extend my special gratitude to Ragnhild Beyrer (former Senior Executive officer and PhD program coordinator) at Department of Community Medicine, Institute of Health and Society, University of Oslo, Norway, for her excellent and unreserved support and encouragement during my study time in Oslo and throughout my study period. I feel home and always cheered with your "nothing is impossible" option to my challenges and it has been a blessing to have you during my study and for my success. I thank you for all the support and love for Ethiopia. I am also grateful for all "Friends of Ethiopia" who planned and visited Ethiopia during the course of this study and for organizing the special Ethiopian occasion in Oslo. I am very grateful for all the staff members of the Department of Community Medicine, Institute of Health and Society, University of Oslo for their unreserved support and encouragement during my study period.

My special gratitude goes to my best friend and colleague, Dr. Mengistu Legesse for his all round support, encouragement and excellent friendship during the challenging fieldwork in Afar and for our best time in Oslo. I am also grateful to Dr. Solomon Yimer, Dr. Mulugeta Belay, Dr. Dawit Shawel and Dr. Demelash Biffa, for their support and encouragement during my stay in Oslo.

I would like to thank the staff of Aklilu Lemma Institute of Pathobiology, Addis Ababa University for all the support and encouragement during the field and laboratory work in Ethiopia. Special gratitude goes to Professor Yalemtsehay Mekonen, Professor Getachew Tilahun, Dr. Mirutse Gidey, Professor Tesfu Kassa, and Dr. Tilahun T/Haimanot for their full support of the NUFU project and establishment and maintenance of successful working collaboration with Afar Nation Regional State Pastoral Bureau and Afar National Regional State Health Bureau. I am very grateful to Dr. Girmay Medhin (Aklilu Lemma Institute of Pathobiology for his scientific contribution in statistical support and comments from study design, data collection, data analysis and interpretation and timely comments on the manuscripts to improve the quality of the study.

I am deeply grateful to ALIPB TB Laboratory staff including Mr. Hailu Getu, Mr. Nega Nigusse, Mr. Adane Worku, Mr. Aboma Zewde, Mr. Tadesse Regassa, and Mr. Bezabih for their excellent support in field research work in the most challenging harsh climatic condition of Afar and in laboratory work at ALIPB. I also extend my gratitude to Mr. Tesfaye Wajo, Girma Kebede, Eshetu and the late Mr. Lakew Nigusse for excellent support and safe driving during the fieldwork in day and night to trace the livestock in pastoral communities.

Special thanks goes to Afar Pastoral and Rural Development Bureau, Afar Health Bureau, Amibara, Chifra, Dubti, and Afambo Pastoral and Rural Development offices, Amibara, Awash Fentale, Gewane Health offices, Afar Regional Veterinary Laboratory, Dubti Referral Hospital, Melka Worer Health Center, Gewane and Awash Health Centers, Melka Worer Agricultural Research Center with all staffs in each offices for their support and facilitations of the research work during data collection, field laboratory work and organizations of field works. I am also very grateful to Afar pastoralists who always were supportive and well coming. My special thanks goes to Mr. Abubaker Mohammed and his family and the whole pastoralist communities in the study sites for their extraordinary support, facilitation and care during our research work, without which this work would have not been successful. You all are special and your exceptional friendship and amazing life experience have shaped me to see things from different dimension.

I would like to thank The Norwegian Institute of Public Health for laboratory support and providing me the necessary training on spoligotype, MIRU-VNTR and HAIN Genotyping techniques and my special gratitude goes to all staffs in Department of Bacteriology and Immunology and National Reference Laboratory for Tuberculosis, to Prof. Dominique A. Caugant, Dr. Turid Mannsaker, Janne Oseberg Rønning, Annika Reichmann, Bente Tora Forsdahl, Dr. Ulf Dahle, Dr. Norheim Gunnstein for their unreserved support and assistance during my laboratory work at Norwegian Institute of Public Health.

I would like to thank Dr. Stefan Berg (Animal Health and Veterinary Laboratories Agency, UK) for his valuable support and contribution for molecular techniques and analysis of Mycobacterial isolates.

I am grateful to College of Veterinary Medicine and Agriculture, Addis Ababa University for the study leave and continuous support of the whole staffs during my study period. I am grateful for Dr. Nesredin Hussen, Dr. Gizachew Baylegne and Dr. Yalelet Work and Dr. Ashenafi Dametew and my entire students who participated in field and laboratory research work and greatly contributed for the success of this work. I thank you the staff and the Department of Biomedical Sciences and Department of Microbiology, Immunology and Veterinary Public Health of the college for their support, encouragement and understanding in sharing my teaching responsibility during the study period. Special thank goes to my friends Dr. Gebeyu Goshu, Melaku Yayehiyirad, Dr. Fekadu Regassa, Dr.Getachew Terefe, Dr. Takele Abayneh, Dr. Yacob Hailu, Dr. Adane Mihret, Samson Abebe, Sileshi Alefom

and all my other friends, not mentioned by names, but certainly not forgotten for their support and encouragement during my study period.

My deepest thanks go to my brothers, Behailu, Henok, Addis, Eshetu and Teshager for your everlasting love, encouragement and support throughout my school life.

Finally, my special gratitude goes to my beloved wife Sara Aklilu for her love, dedication, care, patience and continuous encouragement during those long years of study time fully taking care of family responsibilities. Special thanks to my daughter Bethlehem Gezahegne and my son Esihak Gezahegne for their understanding, support and everlasting love.

ABBREVIATIONS

AAU	Addis Ababa University
AFB	Acid-fast bacilli
AHVLA	Animal health and veterinary laboratories agency
AIDS	Acquired immunodeficiency syndrome
ALIPB	Aklilu Lemma Institute of Pathobiology
ANRS	Afar national regional state
BCG	Bacille de Calmette et Guérin
bp	base pair
BTB	Bovine tuberculosis
CAS	Central Asian
CD	Cluster of differentiation
CDC	Center for disease control
cfu	colony forming unit
CI	Confidence interval
CIDT	Comparative intradermal tuberculin test
cm	centimeter
CM/AS	Common mycobacteria/additional species
CSA	Central statistical agency
DNA	Deoxyribonucleic acid
dNTP	dinucleotide triphosphate
DR	Direct repeat

DTH	Delayed type hypersensitivity
DVR	Direct variant repeat
EAI	East African-Indian
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMbs	Ethiopian *M. bovis* strain
EPTB	Extrapulmonary tuberculosis
ESGPIP	Ethiopia sheep and goat productivity improvement program
ETB	Ethiopian birr
ETR	Exact tandem repeats
FAO	Food and agriculture organization
GDP	Gross domestic product
HBCs	High burden countries
HIV	Human immunodeficiency virus
HPG	Humanitarian Policy Group
IFN-γ	Interferon gamma
IGAD	Intergovernmental authority on development
IS	Insertion sequence
Kb	Kilobase
LJ	Löwenstein Jensen
masl	meter above sea level
MDGs	Millennium development goals

MDR	Multi drug resistant
MIRU-VNTR	Mycobacterium interspersed repeated unit-variable nucleotide tandem repeat
ml	milli liter
MLVA	Multiple-locus variable-number tandem repeat analysis
mm	millimeter
MoARD	Ministry of agriculture and rural development
mPCR	multiplex polymerase chain reaction
MTBC	*Mycobacterium tuberculosis* complex
NCBI	National center for biotechnology information
NMSA	National meteorological services agency
NTM	Non-tuberculous mycobacteria
NUFU	The Norwegian programme for development, research and education
OIE	Office international des epizooties
OR	Odds ratio
PCR	Polymerase chain reaction
PFE	Pastoral forum of Ethiopia
PGRS	polymorphic GC-rich sequence
PPD	Purified protein derivatives
PPD-A	Purified protein derivatives from *M. avium*
PPD-B	Purified protein derivatives from *M. bovis*
PTB	Pulmonary tuberculosis
QFTGIT	Quantiferon TB gold in-tube

QUB	Queen's University Belfast
RD	Region of difference
RFLP	Restriction fragment length polymorphism
RIDOM	Ribosomal differentiation of microorganisms
rpm	revolution per minute
SNNP	Southern nation and nationalities people
SOP	Standard operating procedure
sq km	square kilometer
SSPE	Saline-sodium phosphate- ethylenediaminetetraacetic acid
TAE	Tris-acetate- ethylenediaminetetraacetic acid
TB	Tuberculosis
TST	Tuberculin skin test
UNDP	United nation development programme
WHO	World health organization
ZN	Ziehl Neelsen
χ^2	chi-square

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LIST OF PUBLICATIONS

The thesis was based on the following studies:

Paper I. Mamo G, Bayleyegn G, Sisay Tessema T, Legesse M, Medhin G, Bjune G, Abebe F, Ameni G (2011) Pathology of camel tuberculosis and molecular characterization of its causative agents in pastoral regions of Ethiopia***. PLoS ONE*** 6(1): e15862. doi:10.1371/journal.pone.0015862.

Paper II. Kassa G Mamo, Abebe F, Worku Y, Legesse M, Medhin G, Bjune G, Ameni G. Tuberculosis in Goats and Sheep in Afar Pastoral region of Ethiopia and Isolation of Mycobacterium tuberculosis from Goat. ***Vet Med Int***. 2012; 2012:869146. doi:10.1155/2012/869146. PubMed PMID: 22852105.

Paper III. Mamo G, Abebe F, Worku Y, Hussein N, Legesse M, Tilahun T, Medhin G, Bjune G, Ameni G **(2013)** Bovine tuberculosis and its associated risk factors in pastoral and agro-pastoral cattle herds of Afar Region, Northeast Ethiopia. ***J Vet Med Anml Hlth,*** 5(6), 171-179.

Paper IV. **Mamo G**, Abebe F, Legesse M, Belay M, Worku Y, Mannsaker T, Rønne J, Medhin G, Bjune G, Ameni G (2013) Molecular epidemiology of *Mycobacterium tuberculosis* and isolation of *M. bovis* from pulmonary tuberculosis patients in Afar Pastoral Region of Ethiopia (**Manuscript IV**)

SUMMARY

Ethiopia is one of the 22 high-burden countries with high prevalence and incidence of all forms of tuberculosis in its human population. The country has the largest pastoralist community in East Africa (7-8 million) and these communities are highly marginalized in terms of all basic health services and other infrastructures. Hence, the burden of tuberculosis in these communities is still a serious public health problem in spite of promising progress in recent years across the country. The pastoralists are those communities whose livelihood depends on livestock and migrate from place to place in search of grazing pasture and water for their livestock. The epidemiology of tuberculosis in pastoralist communities is complicated with their socioeconomic setting, cultural habit and their way of life being having close association with livestock. Tuberculosis in human and animal is caused by members of *Mycobacterium (M.) tuberculosis* complex including *M. tuberculosis* and *M. bovis*, which have high genetic similarity at nucleotide level and the diseases caused by these organisms are indistinguishable clinically and/or using acid-fast microscopic examinations at clinic level. In order to understand the epidemiology of tuberculosis in livestock, the role of *Mycobacteria* species from animal origin (zoonotic transmission), and understand molecular genetic diversity, transmission pattern within the communities and among different species of livestock of pastoral area of Ethiopia, molecular epidemiological study is of great importance in pastoralist setting where human-livestock interface is clearly evident. This thesis focuses to address the above research idea in Afar Pastoral Region of Ethiopia and the thesis is based on four papers. The first paper focuses on the epidemiology, pathology of tuberculosis in camels of pastoral regions of Ethiopia and molecular characterization of its causative agents (Paper I), the second paper focuses on the epidemiology of tuberculosis in small ruminants (goat and sheep) of Afar Pastoral Region of Ethiopia and characterization of its causative agents in small ruminants (Paper II), the third paper focuses on epidemiology of tuberculosis in cattle of Afar Pastoral Region of Ethiopia and assessing the associated risk factors for infection in cattle herds of the region (Paper III); the fourth paper focuses on molecular epidemiology of *M. tuberculosis* complex isolated from human pulmonary tuberculosis patients in Afar pastoral region and investigation of the transmission pattern of the causative agents among the pastoralist communities and their livestock. The study revealed a wide distribution with moderately high prevalence of bovine tuberculosis in camel and cattle and low prevalence in small ruminants of the study areas. In camel of Ethiopia, the prevalence of camel tuberculosis was found to be 16.6% based on

comparative intradermal tuberculin test and 10% based on abattoir-based pathological examination. Isolation and molecular characterization of the causative agents of tuberculosis in camel showed that *M. bovis* strain SB0133, *M. bovis* strain SB1953 (new strain reported by this study) and non-tuberculous mycobacteria species including *M. terrae* complex, *M. flavescens*, *M. acapulcensis*, *M. chelonae*, *M. moriokaense* and *M. avium* were the causative agents of tuberculosis in camel causing pathological lesions in the tissues of camels (Paper I). In small ruminants, the prevalence of small ruminant tuberculosis in Afar Pastoral Region was 0.5% at a cutoff ≥4mm and 3.8% at 2mm cut-off point based on comparative intradermal tuberculin test and isolation and molecular characterization of the causative agents from tuberculous lesions showed that *M. tuberculosis* strain SIT149 and non-tuberculous mycobacteria were identified as causative agents in goats. The study revealed the existence of possible transmission of *M. tuberculosis* from human to goats (Paper II). In cattle, the prevalence of bovine tuberculosis was 11% at 4 mm cut-off point and 18.4% at 2mm cut-off point based on comparative tuberculin skin test and age and study districts were found to be risk factors for bovine tuberculin skin test positivity (Paper III). Molecular characterization of isolates from milk and nasal swabs of tuberculin reactor animals identified non-tuberculous mycobacteria including *M. pheli, M. simiae,* and other *Mycobacterium* species, which need further investigation. The molecular epidemiology of *M. tuberculosis* complex isolated from human pulmonary tuberculosis patients of Afar Pastoral Region revealed the presence of high genetic diversity of *M. tuberculosis* (59 spoligotype patterns) with high rate of clustering (75%) indicating the circulation of specific strains in the communities and defect of the tuberculosis control program. Twenty seven of the isolates were new to the spoligotype database (SpolDB4) which were reported by this study. Two of the isolates from human pulmonary tuberculosis were *M. bovis* strain SB1519 indicating the existence of zoonotic transmission of *M. bovis* from livestock to human. Further molecular characterization using MIRU-VNTR 24-loci analysis of the isolates clustered in the spoligotype analysis revealed a high genetic diversity and clustering rate (70%) similarly confirming the existence of recent transmission within the pastoralist communities. This study also reported 16 new MtbC 15-9 strains to the MIRU-VNTR*plus* database (Paper IV-Manuscript).

1. INTRODUCTION

1.1. Human Tuberculosis

Tuberculosis (TB) is a chronic infectious disease of humans and animals caused mainly by the genus *Mycobacteria* grouped in *Mycobacterium tuberculosis* complex (MTBC), which are characterized by 99.95% similarity at the nucleotide level and identical 16S rRNA sequences but differ widely in terms of their host tropisms, phenotypes, and pathogenicity (Boddinghaus *et al.,* 1990; Sreevatsan et *al.,* 1997; Brosch *et al.,* 2002). *Mycobacterium tuberculosis* (*M. tuberculosis*) is the most common cause of human TB, but a considerable proportion of human cases are also caused by *Mycobacterium bovis* (*M. bovis*) (Cosivi *et al.,* 1998; de la Rua-Domenech, 2006), *Mycobacterium canettii* (*M. canettii*) (van Soolingen *et al*., 1997; Pfyffer *et al*., 1998; Miltgen *et al*., 2002), *Mycobacterium africanum* (*M. africanum*) (de Jong *et al*., 2010; Gehre *et a*l, 2013) and other members of MTBC.

TB remains a major global health problem causing high morbidity and mortality among millions of people each year and ranks as the second leading cause of death from an infectious disease worldwide, after the human immunodeficiency virus (HIV) (WHO, 2013). In 2012, an estimated 8.6 million (range, 8.3-9.0 million) people developed TB and 1.3 million died from the disease (including 320 000 deaths among HIV-positive people) (WHO, 2013). Geographically, most of the estimated number of cases in 2012 occurred in Asia (58%) and Africa (27%) and smaller proportions in other regions of the world. The 22 high burden countries (HBCs) accounted for 81% of all estimated incident cases worldwide (WHO, 2013) (Figure 1).

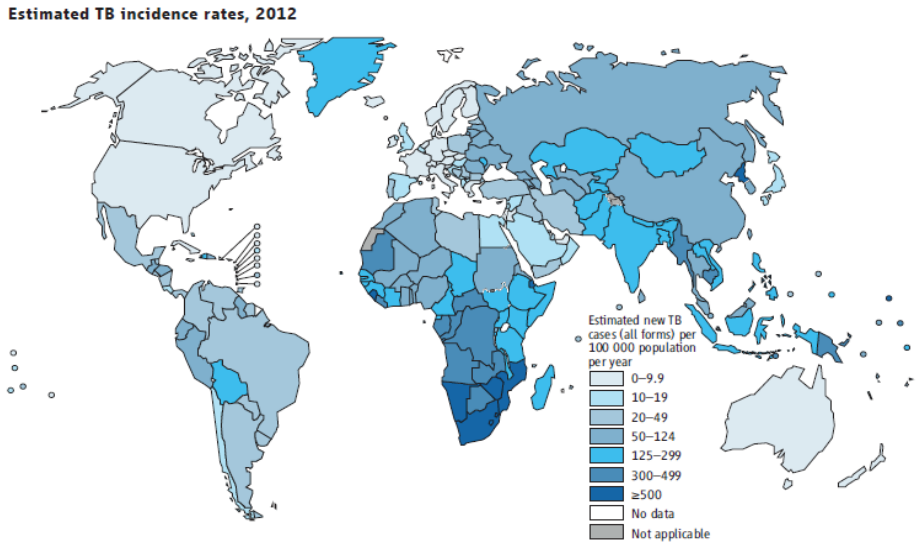

Figure1. Estimated of TB incidence rates, 2012 (WHO, 2013)

Of the 8.6 million incident cases in 2012, 1.0 million–1.2 million (12–14%) were among people living with HIV, with a best estimate of 1.1 million (13%). The proportion of TB cases co-infected with HIV was highest in countries in the African Region; overall, 37% of TB cases were estimated to be co-infected with HIV in this region, which accounted for 75% of TB cases among people living with HIV worldwide (WHO, 2013). In addition, of the total 8.6 million incident cases in 2012, an estimated 0.5 million were children and 2.9 million (range, 2.7–3.1 million) occurred among women (WHO, 2013).

In humans, TB caused by *M. bovis* is clinically indistinguishable from TB caused by *M. tuberculosis*. Globally, the proportion of human TB caused by *M. bovis* is estimated to account for 3.1% of all forms of TB of which 2.1% of pulmonary forms and 9.4% of extrapulmonary forms (Cosivi *et al.,* 1998).

1.2. Bovine Tuberculosis

Bovine tuberculosis (BTB) is a chronic infectious and contagious disease of cattle, other domestic animals, certain wildlife populations, and humans (OIE, 2009). BTB is caused by *M. bovis*, a member of the *Mycobacterium tuberculosis* complex (MTBC) (Mostowy *et al*., 2005) and characterized by the formation of granulomatous lesions in various tissues of

naturally infected animals predominantly being distributed in the upper respiratory tract and associated lymph nodes and also in intestines, liver, kidney and other organs depending on the route of entry of the bacteria (Collins and Grange, 1983; McIlroy *et al.*, 1986; Cassidy, 2006). *M. bovis* has the widest host range among pathogenic mycobacteria mainly spreads within and between animal species via aerosol and ingestion routes (O'Reilly and Daborn, 1995).

In spite of a remarkable genetic identity among members of *M. tuberculosis* complex, particularly *M. bovis* and *M. tuberculosis* there is a distinct host preference of the tubercle bacilli, which can be seen as series of ecotype marked by fixed molecular differences (Smith *et al*, 2006). Epidemiological and experimental data showed that *M. tuberculosis* is highly successful pathogen of human and yet not able to sustain in animal hosts; conversely *M. bovis*, a pathogen of a wide range of animal hosts and rarely transmits between immunocompetent humans (Whelan *et al*, 2010). Immunological analysis of IFN-γ and tuberculin skin test responses clearly showed that both species are equally infective and cause strong cell-medicated immune responses. In experimental analysis of infection of cattle with virulent *M. tuberculosis*, there was a lack of visible pathology and reduced virulence (Whelan *et al*, 2010). However, under natural condition isolation of *M. tuberculosis* from distinct tuberculous lesion of cattle in countries that include India, China, Nigeria and Ethiopia (Ocepek *et al.,* 2005; Cadmus *et al.,* 2006; Chen *et al.,* 2009; Berg *et al.,* 2009) were reported and these may be related to the high burden of *M. tuberculosis* infection in these countries with different genotypes of *M. tuberculosis*, the husbandry conditions and immune status of the cattle or a combination of these factors in the outcome of infection of cattle with *M. tuberculosis* (Whelan *et al*, 2010).

In developed countries, eradication and control programs based on test and slaughter method and pasteurization of animal products have greatly reduced the economic and public health significance of BTB, however, in most developing countries where control program is lacking or not implemented, the disease prevails in wide geographic distribution and has significant economic impact to the livestock sector and creates a zoonotic risk to the exposed human population (Ayele *et al.*, 2004).

1.3. Transmission of Tuberculosis in human and animals

1.3.1. Transmission of *M. tuberculosis* in human

Tuberculosis is transmitted by inhalation of droplet nuclei, which are small, tubercle bacilli containing particles of respiratory secretions expelled into the air when persons with pulmonary or laryngeal TB is coughing, sneezing, or talking. A sneeze can generate 40,000 droplet nuclei and these droplets rapidly evaporated to form droplet nuclei, 1 to 5μm in diameter, which could remain airborne and viable for several days. Inhalation of a single droplet nucleus containing no more than three tubercle bacilli resulted in conversion on tuberculin testing and the development of a macroscopic granuloma in guinea pig (Menzies *et al*., 1995, Griffith and Kerr, 1996, CDC, 2005). In similar way, since these particles can remain airborne for several days or longer, hence individuals can be exposed to tuberculosis even long after the initial source of infection left the site. The tiny infectious particles can be carried by air currents throughout a room or building and inadequate ventilation was found to be an important epidemiological factor for transmission of *M. tuberculosis* (Menzies *et al*., 1995).

M. tuberculosis is usually transmitted through air, not by surface contact (CDC, 2005) hence; there should be an exposure to infectious index case. The probability that a person who is exposed to *M. tuberculosis* will become infected depends primarily on the concentration of infectious droplet nuclei in the air and the duration of exposure to a person with infectious TB disease. The closer the proximity and the longer the duration of exposure, the higher the risk is for being infected (CDC, 2005). The infectiousness of an index case is higher if the patient is not receiving effective therapy (Rouillon *et al.,* 1976), acid-fast bacilli are seen on microscopic examination of a direct smear of sputum, (Rouillon *et al.,* 1976; Loudon *et al*., 1969), radiographic films show a greater extent of disease, or the patient has a frequent cough (Loudon *et al*., 1969).

1.3.2. Transmission of *M. bovis* in animal

Mycobacterium bovis has a wide host ranges that includes most mammalian species. In addition to livestock and wild hoofed mammals, the disease has been reported in elephants, human, non-human primates, and many other species. The ability of *M. bovis* to infect such a wide variety of species can be attributed to the different routes of transmission by which *M. bovis* can transmitted from animal to animal (Thoen *et al*, 2006). There are several routes of transmission for *M. bovis* infection, but the primary routes of infection are via the respiratory

and gastrointestinal tracts (Phillips *et al*., 2003). The nature and extent of tuberculous lesions vary with the route of exposure and the anatomical location of the lesions, which can subsequently, affects how *M. bovis* is excreted from the infected host (Gavier-Widen *et al*., 2001).

1.3.2.1.Respiratory transmission of M. bovis

Respiratory transmission via the inhalation of contaminated aerosols or fomites is the most efficient form of transmission, requiring a low number of organisms as an infective dose (Francis, 1971). Under most circumstances, an infected host generates an aerosol containing *M. bovis* when the animal coughs or sneezes, and the aerosol is inhaled directly by an uninfected host, resulting in infection (Francis, 1971). Under natural conditions, respiratory transmission has been detected in herding animals, such as domestic and wild bovines, and in captive herds of various cervid species (Schmitt *et al.*, 2002). Transmission of *M. bovis* via inhalation is effective in animals that are kept in confinement as in intensive husbandry system (Ameni *et al.* 2007). The high density of cattle, high humidity combined with poor ventilation of the house provide an ideal environment for transmission of the organism, when compared with the environment in which cattle are kept under extensive husbandry system (Phillips *et al.,* 2003; Ameni *et al.*, 2006).

Respiratory transmission of *M. bovis* has been detected in wildlife during periods when normal behaviors become altered and results in direct contact between animals (Schmitt *et al.,* 2002). Evidence for inhalation transmission has been demonstrated in a variety of different species, and lymph nodes associated with the respiratory tract, particularly the bronchial and mediastinal, are the most commonly affected by inhaled *M. bovis* (Palmer *et al.* 2000, Phillips *et al.,* 2003).

1.3.2.2. Oral transmission of M. bovis

Oral transmission through ingestion of *M. bovis* is also important route of transmission next to the respiratory route. Oral route of transmission does not necessarily require close contact of infected animal with uninfected animal. Ingestion of feed or water contaminated with mucous or nasal secretions, feces, or urine that contains the infective bacilli can result establishment of infection in uninfected animals without evidence of direct contact between species (Thoen *et al.*, 2006). The best example for such type of conditions is the cases of *M.*

bovis infection of wild seals in Western Australia where the seals contracted the infection without direct contact with infected animals through consumption of infected cattle carcasses dumped in the sea by some farmers during TB eradication process (Thompson *et al.*, 1993). As in the case of transmission through inhalation, evidence for oral transmission is often found through the distribution of tuberculous lesions in naturally infected animals. In this circumstance, the mesenteric lymph nodes and associated organs are usually affected and have been used to suggest that oral transmission is a more important route of *M. bovis* infection in some situations (Palmer *et al.*, 2000, Gavier-Widen *et al.,* 2001, Phillips *et al.*, 2003).

In extensive animal husbandry system such as that of pastoral production system where animal movement from one area to another and feeding of animals on open grazing pasture are common practices, possibility of contamination of grazing fields by the infective bacilli is the most likely condition and results in transmission of *M. bovis* infection through oral route. A previous study in Ethiopia has demonstrated a predominant localization of lesions in digestive tracts and associated lymph nodes mainly mesenteric lymph nodes in cattle kept in extensive production system (Ameni *et al.,* 2006). This study suggested that oral route of transmission is a common way of transmission of *M. bovis* in animal grazing in open pasture, while respiratory route is the predominant route of bovine tuberculosis in indoor cattle.

1.3.2.3.Other routes of transmissions of M. bovis

Transmission of *M. bovis* including vertical transmission, pseudo-vertical transmission (via milk) and transcutaneous transmissions are some of the less-common and epidemiologically not significant forms of transmission under natural conditions (Thoen *et al.,* 2006). Vertical transmission of *M. bovis* can occur congenitally via the umbilical vessels, as a result of uterine infection of the dam (O'Reilly and Daborn, 1995; Pritchard, 1988) and it has been identified in cattle with very low rate approximately 1% prevalence of *M. bovis* infection of calves in UK (Phillips *et al*., 2003). Pseudo-vertical transmission is possible via the ingestion of tuberculous milk with sub-clinically infected cows typically excreting 10^3cfu/ml of *M. bovis* in milk (Zanini *et a*l., 1998). However, the risks of infection posed by this route of transmission seem to be very small (Phillips *et al.,* 2003).

Another less-common form of transmission is through transcutaneous transmission of *M. bovis*. In animals, transcutaneous transmission is primarily from bites by infected animals.

This has been documented in domestic cats and ferrets (*Mustela furo*) (Ragg *et al.,* 2000), and European badgers (Gavier-Widen *et al.,* 2001). In humans, infection can spread by presence of skin cut and abrasions of skin (e.g., butcher's wart in humans) (Grange and Yates, 1994).

1.3.3. Zoonotic transmission of tuberculosis between human and livestock

In developed countries, where control of bovine tuberculosis in livestock coupled with universal procedure of pasteurization of animal products is practiced the public health importance of human tuberculosis caused by *M. bovis* (zoonotic tuberculosis) is insignificant. However, in the developing countries, zoonotic tuberculosis caused by *M. bovis* is an important public health concern and becoming increasingly prevalent due to the lack of control of animal tuberculosis and widespread consumption of unpasteurized of milk by overwhelming population of these countries (Cosivi *et al.*, 1998). In addition, the high prevalence and incidence of HIV/AIDS in the human population has been also associated with a greatly increased of overt disease in human infected with *M. bovis* (Cosivi *et al.,* 1998). *M. bovis* can be transmitted to humans from a number of mammals, including other humans, and the major potential routes of transmission are gastrointestinal, airborne, and direct contact with mucous membranes and skin abrasions (Thoen *et al*. 2006, Grange and Yates, 1994, Ashford *et al.*, 2001).

The incidence of zoonotic TB associated with oral/gastrointestinal (milk or meat-borne), respiratory (airborne) and cutaneous/mucosal transmission depends on the efficacy of TB control programmes in livestock, food hygiene measures and the habit/practice of the population under consideration (de la Rua-Domenech, 2006). The oral route of transmission is mainly through consumption of unpasteurized milk and milk products which is regarded as the main vehicle for transmission in countries where bovine TB is prevalent and control programmes are patchy or non-existent (Ashford *et al.,* 2001). In these regions, milk-borne *M. bovis* infection is the principal causes of cervical lymphadenopathy and other non-pulmonary forms of human TB (Cosivi *et al.*, 1998). The main source of contamination of raw milk and milk product is the TB infected udder of cow (de la Rua-Domenech, 2006). Excretion of up to 10^3cfu/ml of *M.bovis* has been reported in subclinically infected cows (Zanini *et a*l., 1998). Such large number of *M. bovis* excreted by a single cow with tuberculous mastitis is generally sufficient to render infective the milk pooled from 100 milking cows (Pritchard, 1988). Additionally, milk can be contaminated by exogenous *M.*

bovis bacilli found in dirty milking equipment. *M. bovis* survives well in cows' milk. Viable bacilli can be found in yoghurt and cream cheese made from unpasteurized milk for up to 14 days after preparation and in butter for up to 100 days (Kleeberg, 1984). Hence, ingestion of such contaminated raw milk and milk products are the main route of infection of *M. bovis* in human. With this respect, in pastoralist communities of Ethiopia, milk and milk products originated from cattle, camel, goat or sheep are consumed raw and boiling of milk is a taboo in their culture. Such cultural practice clearly suggests the existence of potential risk of transmission of zoonotic tuberculosis through oral route.

Theoretically, eating undercooked or raw meat and meat products from tuberculous animals could present a mechanism for human infection. However, in practice, transmission of *M. bovis* to humans through the consumption of meat has not been documented as a public health risk during surveillance for TB in many countries. TB lesions in skeletal muscle are very rare and observed only in animals with advanced generalized infection. Whether TB lesions are detected or not, it is possible for apparently normal skeletal muscle of infected cattle to contain bacilli following haematogenous dissemination of *M. bovis* or from the contamination of muscle surfaces during unhygienic dressing of animals with severe TB lesions in other organs (de la Rua-Domenech, 2006). The risk posed by eating undercooked meat of tuberculous animals may be marginally greater in developing countries, where *M. bovis* infection in animals can be quite prevalent but veterinary controls (including meat inspection) are only sporadically applied (Cosivi *et al.*, 1998, Ayele *et al.,* 2004). In Ethiopia, human consumption of raw meat mainly beef is a common and a widespread cultural habit in the population. Hence, such practice can be a potential public health risk for infection with *M. bovis* in human through oral route of transmission.

Respiratory transmission of *M. bovis* from infected animal to human is one of the major potential routes of transmission for contracting zoonotic TB in humans who have close contacts with animals. Several reports support the view that bovine TB can be transmitted from animal to human via aerosol route from infected animals to exposed individuals like abattoir workers, zookeepers, and pastoralists (Georghiou *et al*., 1989, Dalovisio *et al.,* 1992, Gumi *et al.*, 2012). Cutaneous and mucosal transmission routes of *M.bovis* infection of human is extremely rare and occasionally may be seen in those individuals who regularly dressing carcasses of tuberculous animals and veterinarians during surgical interventions or postmortem examinations (Pritchard, 1988; Grange and Yates, 1994; Moda *et al*., 1996; Ashford *et al.,* 2001).

1.4. Diagnosis of bovine tuberculosis in livestock

Diagnosis of bovine tuberculosis (BTB) infection in live animals is usually based on delayed hypersensitivity reactions. Infection is often subclinical when present, clinical signs are not specifically distinctive and can include weakness, anorexia, emaciation in the presence of good nutrition, dyspnoea, enlargement of lymph nodes, and cough, particularly with advanced tuberculosis (OIE, 2009). However, diagnosis based on clinical signs is not conclusive as the signs are not specific to BTB. The most effective methods for diagnosis of BTB are those diagnostic methods carried after death/slaughter of suspected animals. These diagnostic methods include gross postmortem examination (necropsy), histopathology, mycobacteriological culture methods, and various molecular techniques for identification of the isolates. Mycobacterial culture diagnostic method remains the gold standard method for routine confirmation of infection (OIE, 2009).

1.4.1. Tuberculin skin testing

Tuberculin skin tests are the international standard for ante-mortem diagnosis of BTB in cattle herds and individual animals (de la Rua-Domenech *et al*. 2006). Testing for infection in live animals is based on an intradermal reaction to bovine purified protein derivative (PPD), a crude extract of antigens from *M. bovis*. It provides a measure of cell-mediated immunity dependent delayed-type hypersensitivity (DTH) reaction in response to tuberculin (Pollock *et al.*, 2006). Skin DTH is characterized by leukocyte infiltration that is dominated by macrophages and $CD4^+$ and $CD8^+$ T-lymphocytes (Black, 1999).

Tuberculin test may be performed using bovine tuberculin alone as in single intradermal tuberculin test or as a comparative intradermal tuberculin test using purified protein derivative (PPD) of tuberculin from *M. bovis* (PPD-B) and *M. avium* (PPD-A) (OIE, 2009). Animals, which have been sensitized by non-pathogenic environmental strains of mycobacteria, may react positively to PPD-B, due to the presence of antigens common to virulent and non-virulent mycobacterial strains. When this occurs, discrimination between cattle infected with *M. bovis* and those exposed to environmental strains is done using the comparative intradermal tuberculin test (Pollock *et al.*, 2005). The tuberculin test is usually performed on the skin of mid-neck, but the test can also be performed in the caudal fold of

the tail. The skin of the neck is more sensitive to tuberculin than the skin of the caudal fold. To compensate for this difference, higher doses of tuberculin may be used in the caudal fold (OIE, 2009). The single caudal fold intradermal test, the single cervical intradermal test, and the comparative cervical intradermal test are the tuberculin tests used in most bovine tuberculosis control or eradication programs (O'Reilly, 1995; Kaneene and Thoen, 2004).
In spite of its wide use, intradermal tuberculin reactions present some important limitations, related to their sensitivity and specificity (Fra´guas *et al*., 2006). Tuberculin skin test lacks sensitivity and can be confounded by exposure to non-tuberculous mycobacteria, and cannot be repeated for 60 days due to desensitization (Palmer *et al*., 2006). Delayed hypersensitivity may not develop for a period of 3–6 weeks following infection, hence testing using tuberculin test in this period may result false-negative. In addition, tuberculin test may be unresponsive in chronically infected animals with severe pathology (OIE, 2009).

1.4.2. Post-mortem examination

Tuberculosis is characterized by the formation of granuloma (tubercle), which is an organized pathological structure that consists of differentiated macrophages with a characteristic morphology, T lymphocytes, some B lymphocytes, dendritic cells, neutrophils, fibroblasts and extracellular matrix components (Flynn and Chan, 2001; Peters and Ernst, 2003). The complex, dynamic interactions within granuloma lesions reflect a composite of macrophage and helper T cell function, cytokine production and mycobacterial activity that in turn influence the morphological appearance of the granuloma. Lesion necrosis, liquefaction, mineralization and regression represent some of the outcomes of these interactions that dictate lesion size and appearance and ultimately the presentation of disease in the host (Cassidy, 2006).

A presumptive diagnosis of bovine tuberculosis can be made following the macroscopic detection of these granulomatous lesions in different organs of infected animals (Corner, 1994). Macroscopically, a tuberculous granuloma usually has a yellowish appearance and is caseous, caseo-calcareous, or calcified in consistency. Occasionally, its appearance may be more purulent in cervids and camelids (OIE, 2009).

At necropsy, tubercles are most frequently seen in bronchial, mediastinal, retropharyngeal and portal lymph nodes and may be the only tissue affected. In addition, the lung, liver,

spleen and the surfaces of body cavities are commonly affected. In disseminated cases, multiple small granulomas may be found in numerous organs. Early nodular pulmonary lesions can often be detected by palpation (OIE, 2009). Lesion size ranges from small enough to be missed by the unaided eye, to involvement of the greater part of an organ. Serial sectioning of organs and tissues may be required to detect the small lesions contained within the tissue. Non-visible lesion reactors may be due to early infection, poor necropsy technique or infection with mycobacterium other than *M. bovis* (Corner, 1994).

In cattle, tubercles are found in the lymph nodes, particularly those of the head and thorax and abdomen. They are also common in the lung, spleen, liver and the surfaces of body cavities. In disseminated cases, multiple small granulomas may be found in numerous organs. Lesions are sometimes found on the female genitalia, but are rare on the male genitalia. In countries with good control programs, infected cattle typically have few lesions at necropsy. Most of these lesions are found in the lymph nodes associated with the respiratory system. However, small lesions can often be discovered in the lungs of these animals if the tissues are sectioned (OIE, 2008). In general, postmortem examination should be supported by bacteriological examination of lesions for definitive diagnosis of tuberculosis in animals.

1.4.3. Mycobacteriological culture examination

Cultures of mycobacteria require only 10 to 100 organisms to detect *M. tuberculosis* complex. Cultures increase the sensitivity for diagnosis of *M. tuberculosis* complex and allow speciation, drug-susceptibility testing, and, if needed, genotyping for epidemiologic purposes (Brodie and Schluger, 2005). Mycobacteria grow on protein enriched artificial media. There are three types of culture media: solid media, which includes egg-based media (Löwenstein-Jensen) and agar-based media (Middlebrook 7H10 and 7H11), and liquid media (Middlebrook 7H12). The most frequently used solid media is the Löwenstein-Jensen (L-J) containing eggs, phosphate buffer, and magnesium salts, and asparagines (Seifert, 1996). The bacteriological culture differentiation of mycobacteria is based on growth rate, temperature of growth, and production of pigments in light and darkness (Biberstein and Hirsh, 1999) and colony characteristics (Quinn *et al.,* 2004). The surface mycosides (glycolipids and peptidoglycolipids) determine the colony characteristics and serologic specificities (Biberstein and Hirsh, 1999). With regard to the colony characteristics, on solid media *M.*

tuberculosis appears as dry, rough, raised and irregular colony. It is creamy white first and becomes yellowish or buff colored later on and is not emulsified easily. On the other hand, the colony of *M. bovis* is flat, smooth, white and breaking up easily when touched (Gupte, 2006). *M. bovis* can also be identified based on specific biochemical and metabolic properties. *M. bovis* requires pyruvate as a growth supplement and it is negative for niacin accumulation and nitrate reduction and is generally resistant to pyrazinamide. In contrast, *M. tuberculosis* does not require pyruvate as a growth supplement, positive for niacin accumulation and nitrate reduction and is usually not mono-resistant to pyrazinamide (Cole, 2002; Kubica *et al*., 2006).

In the laboratory, *M. bovis* is microaerophilic, i.e. it grows preferentially at a reduced oxygen tension. *M. tuberculosis* is mesophile and neutrophile as its multiplication is restricted to conditions offered by warm-blooded animals: about 37^0C and a neutral PH. The temperature and hydrogen ion concentration ranges, in which the bacillus is able to multiply, are relatively narrow. All the members of the MTBC are slow growers. Therefore, the inoculated media may have to be incubated at 37^0C up to 8 to 12weeks (Quinn *et al*., 2004).

1.4.4. Blood-based laboratory tests

Blood-based laboratory tests are used to confirm or negate the results of an intradermal skin test. Of these tests, the lymphocyte proliferation assay and the gamma-interferon assay correspond to cellular immunity, while the enzyme-linked immunosorbent assay (ELISA) corresponds to humoral immunity (OIE, 2008). Serological assays provide an important tool for testing for exposure to *M. bovis*. Among these tests, gamma-interferon assay is commonly used in conjunction with tuberculin skin testing as a confirmatory test following a positive response to the tuberculin skin test (Palmer *et al*., 2006). IFN-γ diagnostic test is a rapid whole blood assay. The assay is based on the release of gamma-interferon from sensitized lymphocytes during an overnight incubation with a specific antigen. The detection of plasma gamma-interferon is carried out by means of a sandwich-ELISA using specific monoclonal antibodies (Walravens *et al*., 2002). This test in its current form will not generally be acceptable as a direct substitution for skin testing, but could be applied rather as an ancillary test. Benefits of this test include accelerated elimination of tuberculosis from infected herds

and the possibility of the test to be performed as soon as 10 days after the application of a tuberculin skin test. Other applications of the IFN- γ test include confirmation of the immunological status of skin test reactors and the investigation of fraudulent intervention into the skin test (Vordermeier *et al*., 2001).

1.5. Molecular techniques for diagnosis of tuberculosis

A number of molecular based diagnostic methods are available to characterize and type mycobacterial isolates at a specie and/or strain levels. The most common ones used in molecular epidemiology of MTBC include multiplex polymerase chain reaction (mPCR) method (Wilton and Cousins, 1992), region of difference (RD) deletion typing (Huard, *et al.*, 2003), *IS6110*-RFLP typing (van Soolingen, 2001), spoligotyping (Kamerbeek, *et al*., 1997), and MIRU-VNTR 24-loci typing (Supply *et al.*, 2006).

1.5.1. Multiplex polymerase chain reaction (mPCR) method

Multiplex PCR (mPCR) method utilizes the PCR targets on the sequence of the Genus *Mycobacterium* within the 16S rRNA gene (G1, G2) within the hyper-variable region of 16S rRNA that is known to be specific to *M. intracellularae* (MYCINT-F) and *M. avium* (MYCAV-R), and the MTB70 gene specific for MTBC (TB-1A, TB-1B). In this method, it is possible to differentiate all members belonging to Genus *Mycobacterium* and further more characterizes the groups belonging to the *M. tuberculosis* complex and *M. avium* complex. On the gel electrophoresis result all members of the genus *Mycobacterium* produce a band of 1030bp, members of *M. avium* complex produces a band of 850bp (*M. avium* subspecies *intracellularae*) and a band of 180bp (*M. avium* subspecies *avium* and *M. avium* subspecies *paratuberculosis*), while members of *M. tuberculosis* complex including *M. bovis* produces a band of 372bp.

1.5.2. Region of difference (RD) deletion typing

Region of difference (RD) deletion typing is a PCR-based typing method that makes use of the MTBC chromosomal regions of difference deletion loci (Huard, *et al.*, 2003). The

regions of difference represents the loss of genetic material that arise due to errors in DNA replication, movement of mobile genetic elements, mycobacteriophage-mediated transduction, or recombination between adjacent homologous DNA fragments with loss of the intervening sequence (Cole, 2002). Some of these large sequence polymorphisms (LSPs) have been found to be restricted to one MTBC strain or subspecies while others appeared to be differentially distributed among the MTBC groupings. These data have been used to develop a PCR-based method for accurately differentiating several MTBC groupings (Parsons, *et al*., 2002; Huard, *et al*., 2003; Huard, *et al*., 2006). Several PCR primer pairs specific to the loci were used which include; 16S rRNA, Rv0577, Rv1510 (RD4), Rv1970 (RD7), Rv3877/8 (RD1), Rv3120 (RD12), Rv2073 (RD9), Rv1257 (RD13), IS1561 (MiD3) and TbD1 (Huard *et al*., 2003; Huard, *et al*., 2006).

Selective amplification of the 16S rRNA gene was performed on several MTBC and NTM strains and the primers amplified a DNA fragment from all the tested mycobacteria. This gene was therefore chosen to provide the positive control when evaluating mycobacteria by PCR (Huard, *et al*., 2003; Huard, *et al*., 2006). The Rv0577 gene was found to be an MTBC restricted gene. Primers were designed that could specifically and consistently amplify the Rv0577 coding region and this could therefore be used as a genotypic marker for the MTBC and could be used to distinguish the MTBC species from NTM species (Huard, *et al*., 2003; Huard, *et al*., 2006). IS1561 (MiD3) a transposase pseudogene fragment was found to be positive for all MTBC isolates except *Mycobacterium microti* (*M. microti*) (Huard, *et al*., 2003, Huard, *et al*., 2006). Its absence therefore could serve as a good indicator for *M. microti*. Deletion of a 12.7kb Rv1510 gene (RD4) could serve as an indicator for *M. bovis* while it is present in *M.* tuberculosis, *M. africanum* and *M. microti* (Gordon *et al.*, 1999) and on gel electrophoresis *M. bovis* shows a band of 446bp while *M. tuberculosis* shows a band of 335bp. The Rv3877 and Rv3878 (RD1 locus) was selectively absent in *M. bovis* BCG (Huard, *et al*., 2003; Huard, *et al*., 2006). In general, this MTBC PCR typing panel provides an advanced approach to determine the subspecies of MTBC isolates and to differentiate them from clinically important NTM species.

1.5.3. *IS6110*-based restriction fragment length polymorphism (RFLP)

Since the early 1990s, restriction fragment length polymorphism (RFLP) or DNA fingerprinting using the mycobacterial insertion sequence *IS6110* as probe has been used to investigate *M. tuberculosis* transmission within populations. The method is based on the differences in the *IS6110* copy numbers per strain, ranging from 0 to about 25, and variability in the chromosomal positions of these *IS6110* insertion sequences (van Soolingen, 2001). *IS6110* is an insertion sequence belonging to the enterobacterial IS3 family (McAdam *et al*. 1990; Thierry *et al.,* 1990) and it is a 1361 bp long sequence that was detected in members of MTBC and differences of only a few nucleotides have been detected between the sequenced copies. The number of *IS6110* copies present in the genome is species-and strain-dependent. Most strains of *M. tuberculosis* carry eight to fifteen copies in different positions of the genome although single copy strains are common (Kanduma *et al.* 2003). *IS6110*-based RFLP typing is the most widely used method for molecular epidemiological studies because of the high degree of discrimination among different strains of *M. tuberculosis*. For *IS6110*-based RFLP fingerprinting analysis, the DNA from the bacterial culture is first extracted and then is purified. The DNA is then treated with the restriction enzyme *PvuII*; this recognizes a specific six-nucleotide palindromic sequence in the DNA and cleaves it at each occurrence of the sequence. The resulting restriction fragments are separated by electrophoresis based on their size, transferred onto a nylon membrane, and hybridized with an *IS6110* probe. This probe then specifically marks those fragments, which contain the repetitive element *IS6110*. The insertion sequence *IS6110* possesses great variability with respect to the number of the *IS6110* copies (0 to 25 copies) in different strains of *M. tuberculosis* and the number of resulting bands corresponds to the number of *IS6110* copies, and the localization of the bands reflects the molecular weight of the fragments containing the *IS6110* copies. This will help to differentiate the strains of the *M. tuberculosis*. However, *IS6110*-based RFLP has drawback in identifying strains of *M. bovis* and some *M. tuberculosis* strains with one or two *IS6110* copies (van Embden *et al*., 2000), which will be better differentiated using spoligotyping.

1.5.4. Spacer oligonucleotide typing (spoligotyping)

Spoligotyping is a PCR-based molecular method developed to detect and type species/strains of the MTBC (Kamerbeek, *et al*., 1997). The genomes of MTBC carry a single region on the chromosome called the Direct Repeat (DR) locus and DNA polymorphism on this DR locus allow for strain typing. The DR locus spans up to 5 kb and represents 0.1% of the MTBC genome. The MTBC DR region comprises multiple, well-conserved 36-bp DR regions interspersed with non-repetitive spacer sequences varying in length from 34-41-bp (Hermans *et al.,* 1991). One DR and its neighboring non-repetitive space are termed "Direct Variant Repeat (DVR)". *M. tuberculosis* strains vary in the number of DRs, in the presence or absence of particular spacers and the vast majority of the *M. tuberculosis* strains contain one or more *IS6110* elements in the DR region (Groenen *et al.*, 1993) (Figure 2).

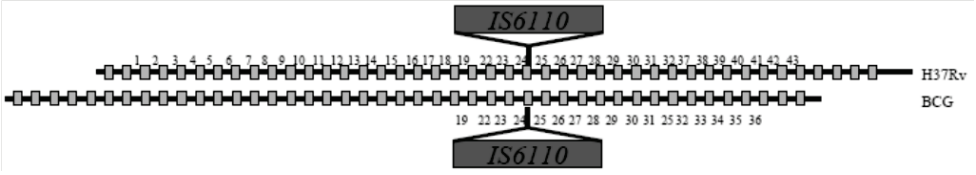

Figure 2. Structure of the DR locus in the genome of *M. tuberculosis* H37Rv and *M. bovis* BCG P3. The small rectangles depict the 36 bp Direct Repeat (DR). (Adapted from: Isogen Life sciences Spoligotype kit manual)

In contrast to the DRs, the spacers are usually present once in the DR region, and more than 100 different spacer sequences have been identified in the MTBC and 43 of them have been selected for use in spoligotyping. When the DR regions of several strains were compared, it was observed that the order of the spacers is about the same in all strains but deletions and/or insertions of spacers and DRs occur. This polymorphism at DVR has been exploited to type and distinguish MTBC strains for epidemiological studies (Kamerbeek, *et al*., 1997; van Embden *et al.*, 2000). In this method, the whole DR region is amplified and labeled by PCR using DR-specific primers and the presence of any of a set of 43 different spacers is determined by hybridization of the amplified DNA to 43 spacer oligonucleotides, which are covalently linked to a membrane. Later detection of hybridization signals is done by the

enhanced chemiluminescence (ECL) detection system and a reaction resulting in the emission of light, which can be detected by autoradiography of the membrane. An example of a result of the spoligotyping method used to analyze a variety of clinical isolates is shown in Figure 3.

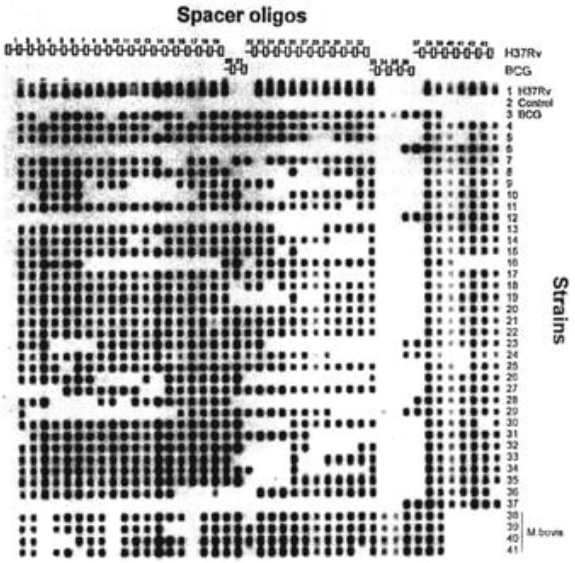

Figure 3. A typical spoligotyping result of *M. tuberculosis* H37Rv, *M. bovis* BCG P3 and 38 different clinical isolates. A membrane with 43 spacer oligonucleotides was used (vertical lines). The spacer oligonucleotides were derived from the spacers of *M. bovis* BCG P3, *M. tuberculosis* H37Rv. (Adapted from: Isogen Life sciences Spoligotype kit manual)

Spoligotyping is a useful method for screening and epidemiologic control of tuberculosis dissemination, particularly when results are required quickly, such as in outbreaks, or in the management of transmission of multidrug-resistant tuberculosis, especially in restricted high-risk situations such as prisons, schools, and hospitals (Gori *et al.,* 2005). Spoligotyping has the advantage of being significantly less technical demanding than RFLP fingerprinting, with a much shorter turnaround time. In addition, the degree of differentiation achieved by spoligotyping is higher than that of *IS6110*-RFLP for *M. bovis,* which usually contain one or two *IS6110* copies (Kamerbeek, *et al*., 1997). One of the major drawbacks to spoligotyping is that it can only identify polymorphisms within the DR cluster, whereas RFLP typing can detect genetic differences arising from multiple loci.

1.5.5. Mycobacterial interspersed repetitive units-variable number tandem repeat (MIRU-VNTR) typing

Mycobacterial interspersed repetitive units-variable number tandem repeat (MIRU-VNTR) typing rely on PCR-amplification using primers specific for the flanking regions of the VNTRs and on the determination of the sizes of the amplicons, after electrophoretic migration. As the length of the repeat units is known, these sizes reflect the numbers of the amplified MIRU-VNTR copies. The result is a numerical code, corresponding to the repeat number in each VNTR locus. Such numerical genotypes are intrinsically portable and are thus particularly convenient for both intra- and inter-laboratory comparative studies (Frothingham and Meeker-O'Connell, 1998; Mazars *et al.*, 2001). Initial VNTR typing systems for MTBC strains made use of very limited sets of loci including exact tandem repeats (ETR) (Frothingham and Meeker-O'Connell, 1998), mycobacterial interspersed repetitive units (MIRUs) (Supply *et al*., 2000; Mazars *et al*., 2001) and sets of Queen's University Belfast (QUB) VNTRs (Roring *et al*., 2002; Skuce *et al*., 2002). MIRU-VNTR 24-loci typing has been proposed for international standardization based on analysis of the clonal stability and evolutionary rates of MIRU-VNTR markers in the genetic lineage of tubercle bacilli collected worldwide (Supply *et al.,* 2006). In addition, the method has been improved as high-speed automated genotyping system with the use of multiplex PCRs for the target MIRU-VNTR loci on a fluorescence-based DNA analyzer with computerized automation of the genotyping (Supply *et al.* 2006).

1.6. Epidemiology of bovine tuberculosis in livestock of Ethiopia

Bovine tuberculosis is distributed throughout the world and in general, the geographic distribution of *M. bovis* in livestock parallels the distribution of livestock throughout the world (Kaneene and Pfeiffer, 2006). A surge in semi-urban dairy industry in many developing countries has increased the risk of epidemic BTB. The majority of people in developing world are directly dependent on their livestock for livelihood; therefore, a failure to control BTB is likely to have a negative impact on the economy and health of the population (Vordermeier *et al.*, 2012). In sub-Saharan Africa, the demographic growth

combined with urbanization and economic development has resulted in the increasing demand for milk, meat and other animal products (Shitaye *et al.,* 2006). The indigenous cattle and the existing extensive production system are unlikely to be able to satisfy the rise in demand for animal products; therefore, intensification of animal husbandry is required, this resulted in increased incidence of BTB. According to OIE database, during the period 1996-2011, the majority of African countries (78%) including Ethiopia reported the existence of BTB in their livestock while only four countries reported the absence of the disease (De Garine-Wichatitsky *et al.*, 2013).

In Ethiopia, the endemic nature of bovine tuberculosis in domestic animals has long been reported (Hailemariam, 1975) and most recent studies also showed that BTB is endemic mainly in cattle in large parts of the country (Ameni *et al.,* 2006, Ameni *et al.*, 2007, Berg *et al*., 2009, Biffa *et al.*, 2011). Majority of the epidemiological studies were carried out on cattle in central Ethiopia and the prevalence reflects variations depending on cattle breeds, husbandry system and other environmental factors (Ameni *et al.*, 2007). The large area of the lowland pastoral regions of country, which harbors about 40% of the total livestock population of the country were not, studied in detail. Hence, it has been difficult to establish the overall national wide epidemiological picture of BTB in Ethiopia. However, review of studies based on tuberculin skin test and abattoir-based examination of the prevalence of BTB in livestock (mainly in cattle) of Ethiopia showed the prevalence ranges from 0.9% to 90% under different management system and breed of cattle. Summary of the data is given in Table 1.

Table 1. Review of the major BTB epidemiology studies in Ethiopia (2003-2013)

Site (Year)	Diagnostic method	Total number of cattle examined	Prevalence (%) (range)	Reference
Central and North Ethiopia (Debre Zeit, Zeway, Sebeta, Sellale, Holleta, Ambo, Wollo)	CIDT	**1168**	**46.8** (10.8-87.1)	Ameni *et al.*, 2003
Central Highland Ethiopia (Selalle, Holleta) (2004-2006)	CIDT (>4mmcut-off)	**5424**	**13.5** (11.6-22.2)	Ameni *et al.,* 2007
BakoGazer, Meskan, Woldia districts (2005-2008)	CIDT (>4mmcut-off)	**5377**	**0.9** (0.3-1.4)	Tschopp *et al.,*2010
Southeast Ethiopia (2009)	CIDT (>4mmcut-off)	**421**	**2** (0.5-8.4)	Gumi *et al.*, 2012
Central Ethiopia (Addis Ababa)	CIDT (>4mmcut-off)	**2098**	**19**	Shitaye *et al.,* 2006
Central Ethiopia (Addis Ababa, Debre Zeit, Sululta, Holleta, Sebeta, Sendafa) (2009-2010)	CIDT (>4mmcut-off)	**2956**	**32.3** (0-90)	Firdessa *et al.,* 2013
Addis Ababa, Gondar, Woldia, Jinka, Butajira Gimbi (2004-2009)	Abattoir -based PM examination	**32779**	**4.7** (1.8-12.7)	Berg *et al.,* 2009
Yabello, Melge Wodo, Addis Ababa. Adama (2006-2007)	Abattoir -based PM examination	**3322**	**10.2** (4.2-24.7)	Biffa *et al.,* 2010
Central Ethiopia (Addis Ababa)	Abattoir-based PM examination	**984**	**3.5**	Shitaye *et al.,* 2006

1.7.Molecular epidemiology of bovine tuberculosis in Ethiopia

The knowledge of molecular epidemiology in the field of tuberculosis have been used to provide novel information about the spread of tubercle bacilli in outbreaks, to track the transmission dynamics of tuberculosis in the population and to distinguish exogenous reinfection from endogenous reactivation. In addition, molecular epidemiology is also being used to identify the source of laboratory contamination, to determine the risk factors for TB transmission in a community, to investigate drug resistance pattern and to track the geographic distribution and spread of clones of mycobacteria species/strains of public health importance (Foxman and Riley, 2001; Narayanan, 2004).

With respect to molecular epidemiology of bovine tuberculosis in livestock of Ethiopia, isolation and molecular characterization of the causative agent for BTB has been carried out in the last decade mainly in cattle and a number of isolates has been reported from different regions of the country. The first *M. bovis* characterized in Ethiopia using spoligotype and VNTR was *EMbs 1 (*Ethiopian *M. bovis* strain 1) named later as SB1176 and its VNTR profile was 5254*33.1 (Ameni *et al.* 2007). This isolate was identified from cattle in Holleta government farm where 17 of bovine tuberculin reactors and with tuberculous lesions were found to be infected with this single strain of *M. bovis*. In 2009, Berg and his colleagues published the result of one of the largest bovine TB studies of its kind in Africa carrying out using postmortem examination on 32,779 cattle slaughtered in abattoirs of different regions of Ethiopia and 135 isolates were characterized using molecular methods, of which 58 were *M. bovis,* 8 were *M. tuberculosis,* 53 were non-tuberculous mycobacteria and 16 were not identified (Berg *et al.* 2009). This work has established important information in term of the molecular epidemiology of BTB and geographic distribution the causative agents of BTB in cattle of Ethiopia (Figure 4).

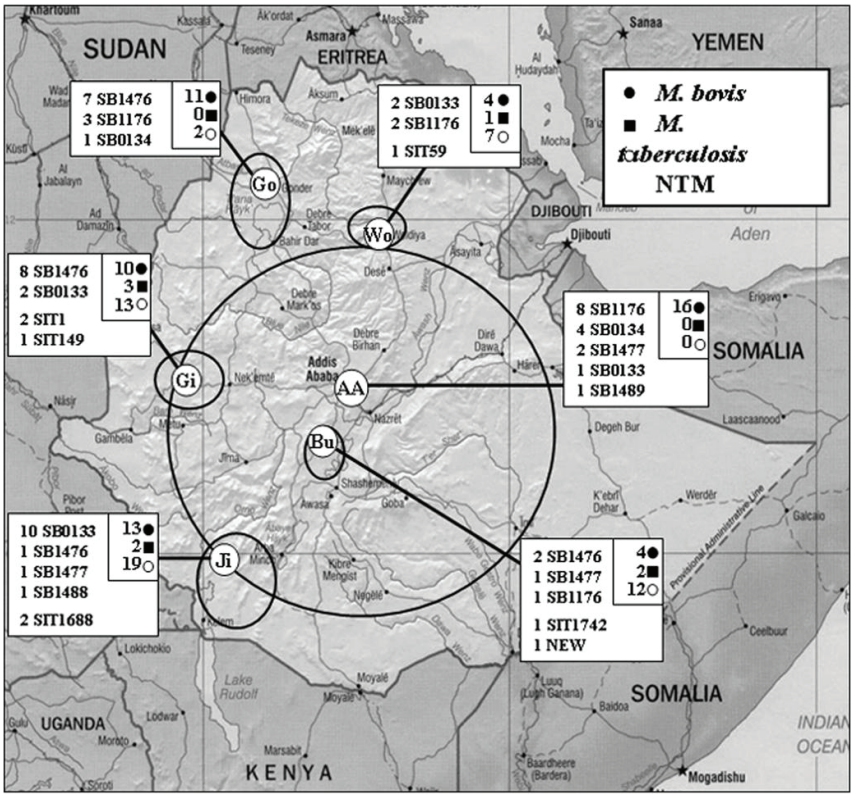

Figure 4**. Geographical distribution of Mycobacterium isolates from cattle in Ethiopia**. The total number of *M. bovis* (●), *M. tuberculosis* (■), and NTM (○), isolated from respective abattoir are indicated in respective box, as well as characterized spoligotype patterns. Approximate area coverage for each abattoir is shown by a solid circle. doi:10.1371/journal.pone.0005068.g001 (Source: Berg *et al*., 2009)

The widespread geographic distribution of *M. bovis* in cattle of Ethiopia and a high degree of genetic polymorphism of the isolates using molecular evidence were further provided with the study carried out in central and southern region of Ethiopia including Borena pastoral zone of Oromia Region (Biffa *et al.*, 2010). This study identified 12 spoligotypes, of which SB1176 was the dominant spoligotype (41.2%) followed by SB0133 (14.7%). New spoligotypes have been reported to www.mbovis.org database including SB1517, SB1518, SB1519, SB1520, SB1521 and SB1522 (Biffa *et al.* 2010). A more recent study in Central Ethiopia on dairy cattle have indicated that SB0134, SB1176, SB0133, and SB1477 spoligotype patterns circulates in the central Ethiopia and further characterization using 24-

MIRU-VNTR showed there is low *M. bovis* strain genetic diversity in the central Ethiopia (Firdessa *et al.,* 2013).

In intensive dairy farms in central Ethiopia, *M. bovis* has been a predominant isolate in dairy cattle (Ameni *et al.,* 2010; Firdessa *et al.,* 2013). Interestingly, a number of studies confirmed that *M. tuberculosis* of different strains as a causative agents of tuberculosis in cattle and other domestic animals particularly in cattle grazing in the field under semi-intensive and/or extensive husbandry systems in central and southeast Ethiopia (Berg *et al.* , 2009; Ameni *et al.* 2010; Gumi *et al.* 2012). Ameni and his colleagues showed that 27% of the isolate from grazing cattle in central Ethiopia were *M. tuberculosis* and the potential route of transmission from human-to-cattle of *M. tuberculosis* was suggested to be the common local practice of farmers spitting chewed tobacco directly into mouths of the cattle (Ameni *et al.* 2010).
On the other hand, a substantial proportion of non-tuberculous mycobacteria were isolated as potential causative agents of tuberculous-like lesions particularly in cattle population under the extensive production system which mainly graze in the field (Berg *et al.,* 2009; Ameni *et al.*, 2010; Gumi *et al*., 2012, Ayana *et al.,* 2013;). Some of the NTM isolated and characterized were *M. avium* subspecies *tropicalis*, *M. intracellulare , M. gordonae, M. arupense, M. holsaticum, M. acapulcensis, M. colombiense, M. engbaekii, M. monacense, M. mucogenicum, M. nonchromogenicum, M. peregrinum, M. vaccae, M. fortuitum* (Berg *et al,* 2009, Ameni *et al.,* 2010, Gumi *et al.,* 2012). These species have been isolated from typical granulomatous tuberculous-like lesions in different tissues including lung, thoracic lymph nodes (bronchial and mediastinal lymph nodes), retropharyngeal lymph nodes, mesenteric lymph nodes and hepatic lymph nodes (Ameni *et al.,* 2010). However, the pathogenesis of these species of mycobacteria and their significance in the epidemiology of animal tuberculosis is not well established.

1.8. Molecular epidemiology of tuberculosis in human patients of Ethiopia

Ethiopia is one of the 22 high burden countries with high annual TB incidence of 247 cases/100,000 population in 2012. Smear positive pulmonary TB, smear negative pulmonary TB cases and extrapulmonary TB cases accounts for 33%, 33% and 33% of all new cases in the country, respectively(WHO, 2013). Isolation of the causative mycobacteria and characterization of these isolates using molecular techniques has an important implication for

understanding the transmission dynamics and assessing the efficiency of the national control program. However, due to poor laboratory resources in the country particularly mycobacterial culture laboratories to isolates the pathogen, very little is known about the causative agents. According to WHO 2013 report, in 2012 the rate of culture laboratories in the country was 0.3 per 5 million populations, which is one of the lowest in world. In spite of these challenges through international collaborations, few studies have tried to elucidate the molecular epidemiology of *M. tuberculosis* isolated from samples collected from human TB patient admitted to health facilities from 1992-2011.

The first molecular epidemiological study of tuberculosis in Ethiopia was done by Hermans and his Colleagues in 1995 on isolates from Addis Ababa pulmonary tuberculosis patients using *M. tuberculosis* specific *IS6110*-based RFLP method, sequencing of PGRS and the direct repeat (DR) regions of *M. tuberculosis* (Hermans *et al.* 1995). On the other hand, a recent molecular study done by Firdessa and his Colleagues was the most extensive molecular epidemiology study of tuberculosis in Ethiopia in terms its wider geographic coverage for samples collections, number of isolates characterized both from pulmonary and extrapulmonary tuberculosis patients and its use of advanced and different types of molecular techniques to characterize the isolates in the study (Firdessa *et al.* 2013). The result of this study was based on molecular characterization of 964 isolates obtained from pulmonary and extrapulmonary patients from different regions of Ethiopia and it showed that a similar distribution of *Mycobacterium tuberculosis* strains between the pulmonary and extrapulmonary manifestations and a minimal role for *M. bovis (*Firdessa *et al.* 2013). This study revealed that out of 964 isolates 954 (98.6%) were *M. tuberculosis*, four (0.4%) were *M. bovis* and 10 (1%) were non-tuberculous mycobacteria (*M. intracellulare*, *M. flavescens*, and *M. simiae*). The *M. bovis* isolated were from southern Borena pastoralist pulmonary patients (Firdessa *et al.*, 2013). This study has also identified a novel new lineage of *M. tuberculosis* (Lineage 7-Woldiya lineage) which is strongly associated with the Horn of Africa, in addition to the already existing six known lineages (East-Asian lineage, Indo-Oceanic lineage, East-African-Indian lineage, EuroAmerican-African lineage, West-African-1 lineage, and West-African-2 lineage).

The *M. tuberculosis* isolates in this newly designated lineage had unusual spoligotype patterns (missing spacers 4-24) and intact for the TbD1 region. Genome sequencing identified these strains as members of a new lineage 7 localized between ancient lineage 1

and modern lineage 2, 3, and 4 of *M. tuberculosis* phylogeny. The new lineage was prominent among strains collected in the Woldiya region Northeast of Amhara Region (Firdessa *et al.*, 2013).

A spoligotype-based molecular study on *M. tuberculosis* isolates from pulmonary tuberculosis patients in Amhara Region of Northwest Ethiopia by Yimer and his Co-authors (2013) described a high genetic diversity of the strains from 237 spoligotyped isolates and identified 65 spoligotype patterns with overall clustering rate of 40%. The predominant spoligotype patterns identified with cluster in this study were SIT25 (14.1%), SIT910 (12.2%), SIT53 (11.4%) and SIT149 (8%); and the T, CAS and U sublineages were the most common sublineages reported in this study (Yimer *et al*., 2013).

Another in-depth study on molecular epidemiology of *M. tuberculosis* and transmission dynamic was carried out on isolates from pulmonary tuberculosis patients in Northwest Ethiopia, which revealed a high genetic diversity of *M. tuberculosis* with 45% of clustering of the strains indicating a high rate of recent transmission in the population (Tessema *et al.*, 2013). This study utilized advanced 24-loci MIRU-VNTR technique and spoligotype on 244 *M. tuberculosis* isolates sampled from pulmonary tuberculosis patients. The Dehli/CAS sublineages were found to be the predominant sublineages (38.9%). In molecular characterization of the strain using 24-loci MIRU-VNTR analysis, the MLVA MtbC15-9 type 594-15 was the predominant cluster forming strain followed by the MLVA MtbC15-9 type 1557-32 strain indicating an ongoing recent transmission of these strains and defect of the TB control program in the region (Tessema *et al*., 2013). Similar recent study carried out on 118 *M. tuberculosis* isolates from smear positive pulmonary tuberculosis patients of Bahir Dar (Northwest Ethiopia) using spoligotyping method showed a high genetic diversity through identification of 36 different spoligotype patterns with 46% clustering of the strains (Debebe *et al.* 2013). In this study, the most predominant spoligotypes were SIT25 and SIT53 consisting of 22/118 (18.6%) isolates and 14/118 (11.9%) isolates, respectively. SIT149, SIT1729, and SIT289 accounted for 7/118 (5.9%) isolates each. The Euro-American African lineage (Lineage 4) was the most common lineage with 64/118 (54%) isolates followed by the Central Asian Linage (Lineage 1) with 37/118 (31.4%) isolates (Debebe *et al.,* 2013).
A molecular epidemiology study carried out on isolates from pulmonary tuberculosis patients of Addis Ababa using *IS6110*-based RFLP DNA fingerprinting method showed that 48% of strains appeared in clusters, and more than half of the clustered strains had less than four

IS*6110* bands (Hermans *et al.* 1995). Secondary typing of these strains by using the sequences of the PGRS and direct repeat (DR) regions as genetic markers decreased the clustering frequency to about 36% (Hermans *et al.,* 1995). A similar other study carried out on 121 *M. tuberculosis* isolates from pulmonary tuberculosis patients in Addis Ababa using RFLP molecular analysis revealed 81 distinct RFLP patterns (1 to 17 *IS6110* copies) indicating the existence of a high genetic diversity of *M. tuberculosis* in study population. The study showed that 42.1% of the *M. tuberculosis* strains were grouped into two major clusters with identical RFLP band patterns and human immunodeficiency virus (HIV)-positive serostatus was significantly associated with clustering of isolates for patients of both sexes (Bruchfled *et al.*, 2002). A high rate of drug-resistant isolates (29.6%) were associated with the peak prevalence of HIV infection in patients 35 to 44 years old and the majority (62.5%) of the resistant isolates in these group were found within clusters indicating the level of recent transmission in HIV-positive population (Bruchfled *et al.*, 2002). This study also characterized two isolates as members of *M. avium* complex as cause of pulmonary tuberculosis, which suggests the important role of *M. avium* complex in the epidemiology of tuberculosis in Ethiopia. None of the isolates with less than five RFLP bands exhibited the specific spoligotyping patterns of *M. africanum*, *M. bovis*, or *M. canettii* (Bruchfled *et al.*, 2002).

A recent molecular epidemiology study on 192 *M. tuberculosis* isolates from Addis Ababa pulmonary tuberculosis patients using spoligotyping also revealed that a high genetic diversity with 53 distinct spoligotype patterns (Mihret *et al.*, 2012). This study also showed that majority of the strains (71.4%) belong two clustered genotypic sublineages: T (49.5%) and CAS genotype (21.9%), however, unlike the previous study there was no significant difference in the distribution of genotype in HIV positive and HIV negative subjects (Mihret *et al*. 2012). In general, these few studies carried out so far on molecular epidemiology of tuberculosis in Ethiopia did not include the major regions including the pastoral regions of Ethiopia which have unique sociocultural and epidemiological setting that can affect the genetic diversity and transmission pattern of different *Mycobacteria* species/strains in human population of the region and hence requires further effort in this respect for efficient control of tuberculosis at a national level. Table 2 shows the review of the major studies on molecular epidemiology of tuberculosis in Ethiopia from 2002-2013.

Table 2. Summary of the major molecular epidemiology studies on *Mycobacteria* species isolated from human tuberculosis patients in Ethiopia (2002-2013)

Study sites (Regions) in Ethiopia	Molecular techniques used	Total number isolates characterized		Types of isolate		Clustering proportion for MTBC	Transmission MTBC	Reference
		PTB	EPTB	MTBC	NTM			
Major Regions of Ethiopia (Addis Ababa, Gondar, Woldiya, Fiche, Negelle/Filtu/Jinka, Gimbi, Butajira)	mPCR, spoligotype, 24-loci MIRU-VNTR, lineage-specific SNP, 16s rDNA sequencing	**629**	**335**	**954**	**10**	64% (PTB) 60% (EPTB)	Recent transmission	Firdessa *et al.,* 2013
Northwest Ethiopia (Bahir Dar and Debre Markos hospital)	Spoligotyping and 24-loci MIRU VNTR	**244**	-	**244**	-	45.1%	Recent transmission	Tessema *et al.*, 2013
Northwest Ethiopia (Felegehiwot Hospital-Bahirdar)	Spoligotyping	**118**	-	**118**	-	46%	Recent transmission	Debebe *et al.,* 2013
Northwest Ethiopia (Amhara Region)	Spoligotyping	**240**	-	**237**	**3**	40%	Recent transmission	Yimer *et al.,* 2013
Central Ethiopia (Debre Berhan)	Spoligotyping, RD9 deletion	**125**	-	**116**	**9**	96%	Recent transmission	Garedew *et al.*, 2013
Addis Ababa (Arada, TekelHaimanot, Kirkos and W-23 Health centers)	Spoligotype	**192**	-	**192**	-	88%	Recent transmission	Mihret *et al.*, 2012
Addis Ababa (Black Lion Hospital)	RFLP and spoligotype	**123**	-	**121**	**2**	**41.2%**	Recent transmission	Bruchfeld *et al.,* 2002

1.9.Pastoralism and role of its livestock in Ethiopia

Pastoralism is a traditional livelihood system based primarily on livestock production for subsistence and characterized by different degree of migration of the community in search of grazing pasture and water for their livestock. Pastoralism is a successful strategy to support a population on less productive land, and adapts well to the environment. It is one of the key production systems in the arid and semiarid dry land of the world (FAO, 2001; HPG, 2009).

Pastoralism most likely developed from agriculture as people migrated into areas of low productivity and/or regions of unreliable rainfall (Weber and Horst, 2011). As a result, these people came to rely upon domesticated animals for subsistence instead of agricultural crops (Salzman, 2004). The most typical forms of pastoral production are transhumance and nomadic forms (Yalcin, 1986). Transhumance includes the seasonal movement of animals and people from valley bottoms to mountain pastures (Yalcin, 1986) while nomadic pastoralism may have developed in response to recurring and widespread drought and is typified by livestock being moved in constant search for forage (Salzman, 2004). Nomadic form differs from transhumance in that no permanent base (home or village) is developed and likewise, no pre-defined series of movements are used. In pastoralism, mobility is a key strategy used by pastoralists to utilize available resources, notably pasture and water (Kaimba *et al*., 2011).

Pastoralism covers about 25% of the earth's terrestrial surface, and it is an important economic and cultural way of life for about 200 million people throughout the world surface (WISP, 2008). In developing countries, pastoralism accounts for the livelihoods of 50 - 100 million people and approximately 60% of this population lives in more than 21 African countries confined to the most arid regions of the continent (Sheik-Mohamed and Velema, 1999; UNDP, 2007).

In East Africa, Ethiopia has the largest pastoralist population (7-8 million), representing around 20 ethnic groups and constitute around 14-18% of the total Ethiopian population (Markakis, 2004). Pastoralists in Ethiopia are found in seven regions including Afar, Somali, SNNP, Oromia, Dire Dawa, Benshangul Gumuz and Gambella Regional States. The major

ethnic groups in Ethiopia are Somalis, Afar, Kereyu and Borena pastoral communities occupying the Eastern, Northeastern and southern lowlands of the country (PEF, 2010a).
Ethiopia's total livestock population has reached more than 88 million in head count, and is the largest in Africa (MoA, 2010; Shitarek, 2012). The livestock sub-sector contributes an estimated 12% to total GDP and over 45% to agricultural GDP (Shitarek, 2012). On average, the pastoral livestock population accounts for an estimated 40% of the total livestock population of the country (Pantuliano and Wekesa, 2008) and the sector plays a crucial role for livelihood of the pastoralist communities. IGAD estimated in 2010 that pastoralist livestock makes up 30% of the nation's cattle, 70% of the goats and sheep and all camels in the country (PFE, 2010b; Shitarek, 2012). The pastoral population occupies a disproportionately large area of Ethiopia and produces much more than its share of national livestock output. The Ministry of Agriculture estimates that pastoralists use 60% of the country's land area, though exact figures of the pastoral livestock population in Ethiopia are unknown(MoARD, 2010).

In 2010, IGAD commented that the contribution of pastoral livestock to the national GDP had been underestimated in previous years. However, according to a revised formula to value livestock assets to the national economy, IGAD estimated that pastoralist livestock contributed 35 billion Ethiopian Birr (ETB) (around $ 2 billion) out of the total national livestock value of 86.5 billion ETB (around $4.8 billion) to the national economy for 2008/09 (IGAD, 2010). According to IGAD, the pastoral livestock population also contributes to transport services in pastoral areas and provides products such as milk, meat, skin and hides, also involved in livestock sharing networks as a collective insurance value, though the value of these components has largely been underestimated. In general, considering all these values, IGAD estimated that the contribution of pastoral livestock to Ethiopia's GDP is very significant and exceeds 90 billion ETB, approximately US$10.6 billion in 2008/09. More than the economic value, to the pastoralist community livestock shares the core of their cultural identity, wealth ownership and means of subsistence in the midst of the hostile and challenging climatic and ecological settings of the arid zone.
As in most developing countries, pastoralist in Ethiopia have been still marginalized in terms of basic infrastructure including access to basic education, health services to both human and their livestock, and access to communication in spite of some efforts in the past decades by government and non-government organization. Hence, the pastoralist livelihoods are highly vulnerable to the effect of climatic changes including the recurring drought and epidemics of

diseases affecting livestock and humans with huge negative impact on their survival, animal productivity and public health of the pastoralist population (Perry *et al.*, 2002).

1.10. Tuberculosis in livestock in pastoral regions of Ethiopia and its zoonotic significance

1.10.1. Tuberculosis in cattle

Few studies have been carried out on tuberculosis in cattle (classical BTB) of Borona and Hamer Pastoral areas of Southern Ethiopia with a prevalence of 0.8% (Tschoop *et al*., 2010) in cattle of Hamer pastoral district of SNNP Region and 5.5% (Gumi *et al.*, 2011) of prevalence in cattle of Borona Pastoral zone of Oromia Region using CIDT method. An abattoir-based study carried out in Yabello Abattoir on cattle of Borona pastoral area found a 4.2% prevalence of BTB (Biffa *et al.*, 2009). Regarding the identification of the etiology of BTB, different strains of *M. bovis* and NTM including *M. fortuitum, M. avium* complex, *M. terrae* complex were characterized and confirmed using molecular techniques as causative agents in cattle of pastoral region studied (Gumi *et al.,* 2012; Biffa *et al*., 2010).

1.10.2. Tuberculosis in small ruminants

In spite the important role of small ruminants for skin, meat and milk production in Ethiopia, little information is available on the epidemiology of caprine tuberculosis in the country. It has been reported that prevalence of TB in Ethiopian goats was 4.2% and this was the first report on the occurrence of the disease in goats of Ethiopia (Hiko and Agga, 2011). This study have also reported the isolation of *M. bovis and M. tuberculosis* from slaughtered goats at Modjo abattoir with tuberculous lesions, however, their diagnosis were based only on colony morphology and discrimination by culture on growth media with pyruvate or glycerol supplements (Hiko and Agga, 2011) and hence the isolation were not confirmatory.

The study of caprine tuberculosis conducted on randomly selected 630 goats at Adami Tulu Agricultural Research Center using single intradermal tuberculin test recorded prevalence of 3.1% (Tafess *et al.,* 2011). While study conducted on goats and sheep of central Ethiopia, using comparative intradermal tuberculin test (CIDT) recorded 0.41% at 2mm cut-off point (Tschopp *et al.,* 2011).

In livestock of Somali pastoral region in south east Ethiopia, a cross-sectional study of bovine TB detected by the CIDT recorded prevalence of 0.2% in goats (Gumi *et al.,* 2012) and in this study further isolation of the causative agent recovered nine NTM (*M. terrae* complex and *M. arupense*) from tuberculous lesions (Gumi *et al.*, 2012). A recent study conducted on 1990 randomly selected male goats that were slaughtered at Luna Export abattoir of central Ethiopia using post mortem examination, mycobacterial culturing and molecular typing techniques also showed 3.5% prevalence of caprine TB. However, the studies identified four isolates to be members of the MTBC, of which three of them were *M. tuberculosis* strain SIT53 on spoligotyping (Deresa *et al.,* .2013). In Hamer pastoral district of south Ethiopia, out of the 186 tested using CIDT none was found to be positive for tuberculosis (Tschopp *et al.*, 2010).

1.10.3. Tuberculosis in camels

Ethiopia possesses one of the largest camel populations in Africa with around 1.07 million of dromedary camel (*Camelus dromedarius*) which are exclusively localized in pastoral region of the country (CSA, 2008). The camel is a versatile animal capable of living in harshly semiarid and arid areas of the world and it is extremely important for livelihood of pastoral communities through provision of milk, meat and draft power for transportation of goods. In pastoral communities of Afar, Somali and Borena, camels are kept almost entirely for milk production (Getahun and Belay, 2002). In these communities, camel milk is consumed raw, and this habit combined with close physical contact with their animals create a potential public health concern for transmission of zoonotic diseases such as tuberculosis (TB) from animals to the pastoralist.

In general, very little is known about the epidemiology and the causative agents of camel TB in pastoral areas of the world. In Ethiopia, few reports exists on camel TB since its first reported in 2009 (Mamo *et al.,* 2009; Gumi *et al*, 2012) and still there is a large paucity of information on its causative agent, epidemiology, risk factors and its zoonotic significance for the pastoralist communities of Ethiopia. In the first abattoir-based camel TB study from eastern pastoral areas of Ethiopia 5.07% prevalence of camel TB was reported. The study also confirmed that members of *M. tuberculosis* complex as a causative agent based on culture and multiplex PCR (Mamo *et al.*, 2009). Gumi and his Colleagues has characterized

M. tuberculosis strain SIT149 using spoligotype from disseminated generalized TB cases of camel and NTM as a causative agents of camel TB in south east pastoral camels of Ethiopia (Gumi *et al.*, 2012).

1.10.4. Zoonotic significance of tuberculosis and risk factors for transmission in pastoral communities

Although human tuberculosis is caused mainly by *M. tuberculosis*, *M. bovis* the etiological agent of bovine tuberculosis can also be responsible for human tuberculosis, which makes this bacterium an important zoonotic species (Beit *et al.*, 2005). *M. bovis* has the broadest host range, actually the largest of any member of the *M. tuberculosis* complex. It causes disease in a wide range of domestic but also free-ranging and farmed wildlife animals as well as in humans (Beit *et al.*, 2005). Clinically, human TB caused by *M. bovis* is indistinguishable from TB caused by *M. tuberculosis* and it requires laboratory facilities for culture-based isolation and molecular typing of the tubercle bacilli (de Kantor *et al.*, 2010), which is rare in most developing countries like Ethiopia.

In countries where BTB in cattle is still highly prevalent and pasteurization is not widely practiced and/or milk hygiene is insufficient, usually estimated to be about 10% to 15% of human tuberculosis is considered to be caused by *M. bovis* (Ashford *et al*., 2001). Human tuberculosis caused by *M. bovis* has been confirmed in a number of African countries including in Egypt in pulmonary TB cases (0.4-6.4%) (Elsabban *et al.*, 1992) and in extrapulmonary cases of TB peritonitis (0.45%) (Nafeh *et al.*, 1992), in Nigeria *M. bovis* has been isolated both from pulmonary TB and extrapulmonary TB human cases (Cadmus *et al.*, 2006), in Tanzania *M. bovis* has been isolated from 16% of cervical adenitis cases of extrapulmonary TB (Kazwala *et al.,* 2001), in Uganda from cervical lymphadenitis case from pastoral communities 7% of the cases were due to *M. bovis* (Oloya *et al.,* 2008).

In Ethiopia, human tuberculosis caused by *M. bovis* has been reported in both pulmonary tuberculosis and extrapulmonary cases. The proportions *M. bovis* isolated and characterized were around 2% in pulmonary TB patients of pastoral communities of Borona area, south Ethiopia (Gumi *et al.*, 2012) and 17% in extrapulmonary TB lymphadenitis cases from Butajira area in central Ethiopia (Kidane *et al.,* 2002); later other study carried out in the

same Butajira and other urban areas of Ethiopia, no *M. bovis* has been isolated from fine-needle aspirates of TB lymphadenitis cases (Beyene *et al.*, 2009) creating some form of controversy on the issue. These studies utilized advanced molecular techniques to differentiate the *M. bovis* isolates from *M. tuberculosis*, which makes it as reliable results as compared to other previous reports in Ethiopia, which used culture growth characteristics and biochemical test to differentiate the species (Kiros, 1998; Regassa *et al.,* 2005, Fetene *et al.,* 2011).

The proportion of which BTB contributes to the total of tuberculosis cases in humans can be affected by the epidemiology of BTB in livestock and existence of potential risk factors for transmission of *M. bovis* from animal to human. In Ethiopia, the pastoralist communities who entirely depend on livestock for their subsistence have special association with their livestock. Livestock of different species including cattle, goat, sheep, camel are reared in large herd size, pastoralists have very close physical contacts during milking, herding, watering and also in night small ruminants and calve may spent in the same hut (Figure 5) together with their owner, and based on the socio-cultural habits, most pastoralist communities of Ethiopia consume animals products specially milk and other animal by-product from all species in raw (without boiling/pasteurization). The existence of such risk factors indicate the potential risk of high rate of exposure of the pastoralist communities for zoonotic tuberculosis transmission caused by *M. bovis* and also for the possibility of occurrence of reverse zoonosis in which *M. tuberculosis* from human patients can infect domestic animals.

Figure 5: Afar House made of wood scaffold and roof covered with straw-made cover

In spite of the presence of risk factors for transmission of zoonotic tuberculosis from animal to human or vice versa in pastoral area of Ethiopia, so far there are only few published works; of which one was carried out in south east pastoral Borena area of Ethiopia (Gumi *et al.,* 2012). According to this study, from a total of 161 isolates from sputum (pulmonary TB) and fine-needle aspirate (extrapulmonary TB) samples, three were confirmed to be *M. bovis* (1.9%) from sputum sample of pulmonary TB patients (Gumi *et al.* 2012). These three *M. bovis* has a spoligotype pattern of SB0133 and SB0303 strains. The study also isolated the same SB0133 strain from cattle in the same area indicating the zoonotic transmission of the strain in the pastoralist communities of south east Ethiopia. Previous abattoir-based study on cattle in Yabello area (South east Ethiopia) characterized SB0133 strains in cattle (Biffa *et al.*, 2011).

1.11. The Rationale of the study

Tuberculosis remains a major global health problem causing high morbidity and mortality among millions of people each year and ranks as the second leading cause of death from an infectious disease worldwide, after the human immunodeficiency virus (HIV) (WHO, 2013). In Africa, Ethiopia with the its 86, 613, 986 million human population (CSA, 2013), is the second most populous country in Africa next to Nigeria and it is one of the 22 high-burden countries with an estimated prevalence of all forms of TB 224 per 100 000 population and incidence rates of 247 per 100 000 population (WHO, 2013). In spite of a promising progress in scaling-up access to the basic health services including the national TB control activities all across the country, marginalized pastoralist communities still have the poorest access to health services, insufficient laboratory facilities and short of trained manpower, to this effect the burden of tuberculosis in these communities is still a serious public health problem.

The Afar pastoralist communities in northeastern Ethiopia are one of the most marginalized communities in terms of health services facilities and are highly affected by TB. According to the WHO 2009, the Afar Region reported a notification rate between ranging from 146 and 260 per 100,000 populations, which is one of the highest notification rates in Ethiopia in 2007 (WHO, 2009). A recent community-based study carried out on the epidemiology of tuberculosis in Afar pastoral region indicated that the prevalence of latent tuberculosis in the population was 63.7% using QFTGIT and 31.2% using TST, which suggest the potential reservoir of latent infection which can progress to active tuberculosis cases (Legesse *et al.*, 2011) in the presence of number of immunocompromising risk factors like malnutrition and HIV infection in the region. In other similar study, large numbers of undetected active pulmonary TB cases (27.9%) were confirmed using mycobacterial culture and multiplex PCR in the general population (Legesse *et al.*, 2013) of the studied district in Afar region. Hence, tuberculosis is a major public health problem in Afar pastoralist community. However, so far no in-depth study have been carried out to identify and characterize the specific mycobacterial etiology of human tuberculosis in the Afar pastoralist TB patients using advanced molecular technique in order to understand the genetic diversity and transmission patterns of circulating species/ strains of *M. tuberculosis* complex and other *Mycobacterium* species isolated from pulmonary patients of Afar Region.

On the other hand, bovine tuberculosis has been known to be endemic in Ethiopia in majority of the highland livestock population; however, very few studies were carried out in the lowland pastoralist region of the country, which constitutes about 40% of the livestock population of the country. The Afar Pastoral region which is located on the Northeast part of Ethiopia possesses huge livestock resource (10,179,277) composed of different species of livestock, of which 4,267,969 (goat), 2463632 (sheep), 2336483 (cattle), 852016 (camel). The density of animal per square kilometer areas varies from area to area within the region and it ranges from 6044 animals /sq km in Zone Two to 31006 animals /sq km in Zone Five (ANRS, 2006). Afar pastoralist are known for rearing different species of animals including camel, cattle, goat and sheep in large numbers and close relatives or families keep their animals together.

In spite of these huge livestock resources of in the region and existence of potential risk factors for transmission of mycobacterial infections, there was no study on the epidemiology of tuberculosis and associated risk factors for infection in different species of livestock (cattle, goat, sheep and camel) in Afar Pastoral Region Ethiopia. As the same time, in majority of Pastoral Regions of Ethiopia, including Afar Pastoral Region, very little/no information are available on camel and small ruminant tuberculosis related to its pathology, species of causative agents and their molecular genetic diversity of the causative mycobacterium species. Hence, addressing these gaps of knowledge have critical importance in understanding the epidemiological distribution, the genetic diversity of the causative mycobacterial species and their circulation pattern in different species of livestock and help to design feasible BTB control strategy in Afar Pastoral Region. In addition, the research also helps to elucidate the public health significance of bovine tuberculosis in pastoral region and its transmission pattern to the pastoralist communities for designing a control options to reduce the public health risk of transmission to pastoralist communities.

2. OBJECTIVES OF THE THESIS

2.1. General objectives

- To investigate molecular epidemiology of *Mycobacterium tuberculosis* complex in Afar pastoral communities and their livestock
- To study the epidemiology of tuberculosis in cattle, goat and sheep, and camel of Afar Pastoral Region of Ethiopia.

2.2. Specific Objectives

- To assess the pathology of camel tuberculosis and molecular characterization of its causative agents in camel of Ethiopia (Paper I)
- To assess the prevalence of tuberculosis in small ruminants (goat and sheep) and its associated risk factors and molecular characterization of the causative agents in Afar Pastoral Region (Paper II)
- To assess the epidemiology of bovine tuberculosis and its associated risk factors in cattle of Afar Pastoral Region (Paper III)
- To assess the molecular epidemiology of *M. tuberculosis* isolated from pulmonary patients of Afar Pastoral Region and investigate the zoonotic transmission of *Mycobacteria* species from animal to human and vice versa (Manuscript and Paper II)

3. MATERIALS AND METHODS

3.1. Description of the Study Region and districts

3.1.1. Study Region

Ethiopia is located in the horn of east Africa and is bordered by Kenya, Somalia, Sudan, South Sudan, Eritrea and Djibouti. The country has a total population of 86 million in an estimated total area of 1, 127, 127 square kilometers and the second most populous country in Africa next to Nigeria (CSA, 2013). The country is divided administratively into nine regional state and two city administrative councils (Addis Ababa and Dire Dawa). Each regional state is further divided into zones, districts ("woreda"), sub-districts ("kebeles"). "Kebele" is the lowest administrative unit in Ethiopia, with an average population size of 5000 in rural areas and 25000 in urban areas (MoH/EHNRI, 2011).

The Afar Pastoral Region is one of the nine regions of Ethiopia and located in northeast of the country between 39° 34' to 42° 28'E longitude and 8° 49' to 14° 30' N latitude. The region shares common international boundaries with Eritrea in the north-east and Djibouti in the east, and it is characterized by an arid and semi-arid climate with low and erratic rainfall. Rainfall is bi-modal throughout the region, with a mean annual rainfall below 500 mm in the semi-arid western escarpments and decreasing to 150 mm in the arid zones to the east. The altitude of the Region ranges from 120 m below sea level in Danakil depression to 1500 m above sea level. Temperatures vary from 20°C in higher elevations to 48°C in lower elevations. The human population of Afar region is 1, 411, 092 million in which the majority (90%) are pastoralists who largely depend on livestock production for their livelihood and the population density of the region is estimated to be 14.6 persons per square kilometer though it varies from zone to zone (CSA, 2007; ANRS, 2010). The Afar National Regional State has an estimated area of 96, 707 square kilometer and is divided into five administrative zones, 32 districts ("Woredas"), and 358 sub-districts ("Kebeles"). From these five administrative zones, zone one is the largest zone, it covers 34.58% of the regional area were as zone five is the smallest zone in the region, it cover 5.78 % of the region area (ANRS, 2006). The Afar Region has two major rivers (Awash and Telalak Rivers) which form their river basin (ANRS, 2006). The Awash river which the major basin forming river originating from central Ethiopian highlands, flows across the region from south to north to enter to Lake Abe at the

Djibouti border. The bank of Awash River is the main site where livestock migrate during dry seasons of the year. The Awash River basin is divided into Upper Awash Basin (from Koka Dam in central Ethiopia to Awash Station in Awash district and has altitude between 1500-1000masl); Middle Awash Basin (from Awash Station to Gewane town which has an altitude between 1000-500masl) and Lower Awash Basin (from Gewane town to Lake Abe which has altitude below 500masl).

As part of a major project entitled "Studies of molecular epidemiology, clinical epidemiology, and immunology of tuberculosis in pastoral communities and their livestock in Ethiopia" funded by NUFU2007/11 programme, for this PhD project five districts (Awash Fentale, Amibara, Dubti, Chifra and Afambo districts) were selected for molecular epidemiology of *M. tuberculosis* complex study on isolates collected from pulmonary TB patients of Afar Region and for animal tuberculosis epidemiology study on cattle, goat, sheep and camel using comparative intradermal tuberculin test four districts (Amibara, Dubti, Chifra, and Afambo districts) were selected based on the population of livestock, accessibility to their respective subdistricts and existence of relative peace in the districts (Figure 6).

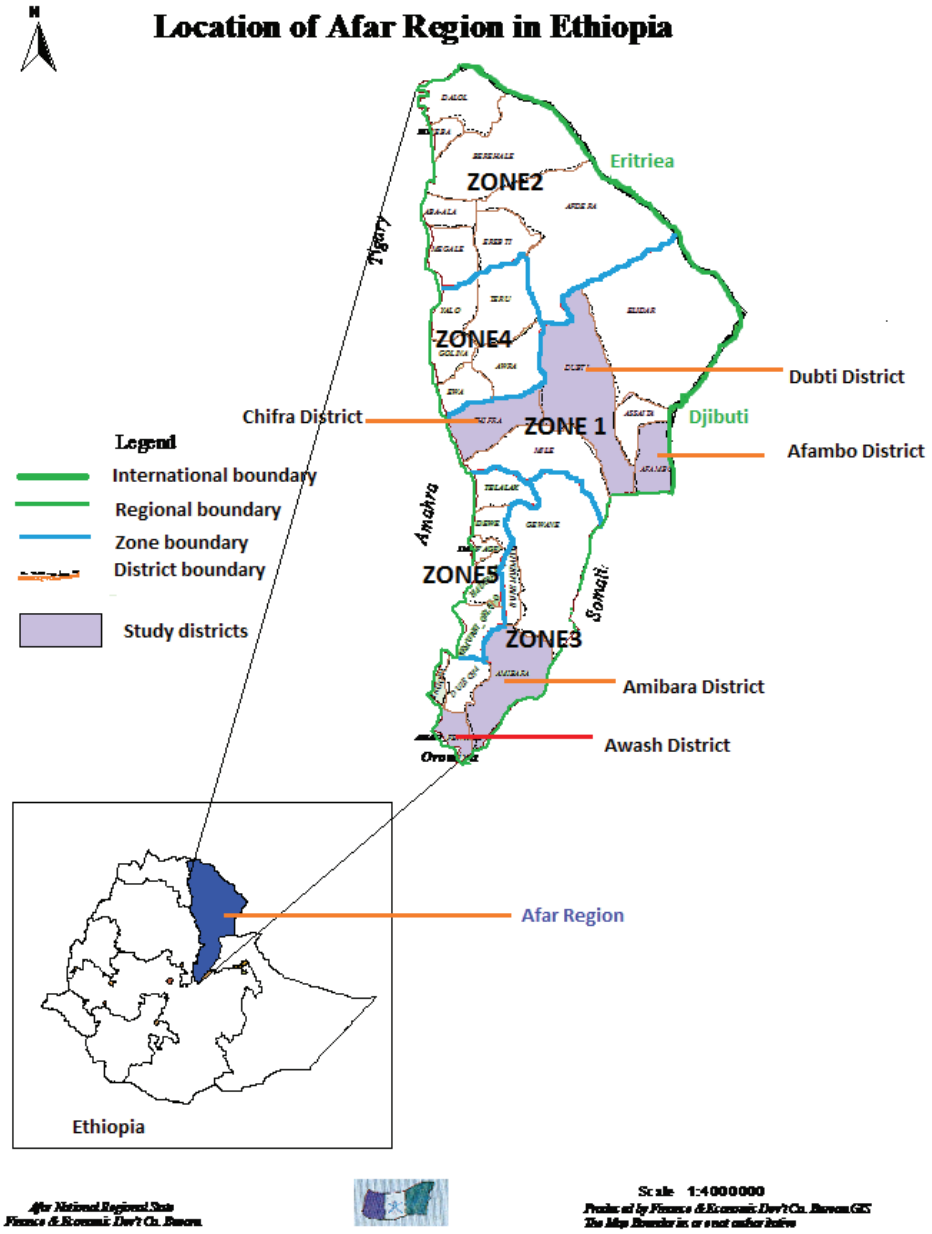

Figure 6: Location of Afar National Regional State in Ethiopia and study districts

For abattoir-based camel tuberculosis study, Metehara Abattoir and Akaki (in Addis Ababa) Abattoir were used to study the TB pathology, isolation and molecular characterization of the isolates collected from camels originated from two main pastoral regions of Ethiopia, namely Awash-Fentale pastoral area (Kereyu area from Oromia region and Afar region the Middle Awash basin) and Borena pastoral area (Southern Ethiopia). The catchment areas possess large number of camels, in Awash-Fentale pastoral area (Middle Awash region) there are 68,331 camels and in Borena pastoral area of Oromia Regional State which border with Kenya possesses an estimated population of 97,131 camels (CSA, 2008).

3.1.2. Study Districts

The animal tuberculosis study on cattle, camel, sheep and goat was conducted in Dubti, Afambo, Amibara, Chifra districts of Afar National Regional State.

3.1.2.1.Amibara District

Amibara District is one of the districts in Zone three of Afar National Regional State which is located in the Middle Awash Basin about 260km to north east of Addis Ababa. Amibara District is bordered on the south by Awash Fentale District, on the west by the Awash River, which separates it from Dulecha District to the southwest then on the northwest by the administrative Zone 5, on the north by Gewane, and on the east by the Oromia Region. The area is characterized by high temperature; it ranges from 25^0C to 35^0C. Usually the mean annual precipitation is less than 600 mm. May/June is the driest season of the year, *hagay*. It is said to be unsuitable for browsing since bushes dry up. The main rainy season *(Karima)* which extends from July to September accounts for above 60% of the annual total rainfall. The district has 18 *kebeles* of which four are in towns, while the rests are pastoral *kebeles.* The district has a total human population of 68,146 of which the majority of the inhabitants are pastoralist. Melka Werer Health Center is the main health facility for the district and now a new hospital has began its service to the population in the district and surrounding districts in the zone. The livestock populations of the Amibara District are composed of cattle 103, 959, goats 122, 526, sheep 48,043, donkeys 3,888 and camels 39,995(CSA, 2005). A family that has no camel is considered poor, regardless of other livestock, and those who have at least one camel are considered rich. The wealthy families who have more than 30 camels are very respected by the society in the area.

Figure 7. Large herd size and mixing of different species of livestock in Amibara district

The pastoralists in Amibara District have large herd size composed of different species of animals (camel, cattle, sheep and goat) (Figure 7) and spend most of the wet season in *Halidege* plain ("*Adadi"* pasture land) where all the Afar clans in the district evacuate their livestock for grazing and in dry season along Awash river bank. During the main rainy season the flood hazard displace the people away from the Awash River. Because of the presence of large pasture land and rivers in Amibara districts, animals from different districts also migrate to Awash River banks and vast pasture lands where intermixing of different species and herds of livestock occur. In Amibara District, there is a large private-owned cotton farms, which after harvest, become grazing sites where large number of herds of different species of livestock from various districts congregate to graze on the leftovers of the harvest creating a potential risk factor for interspecies and interherd disease transmission. The Amibara District has also "Adadi" wildlife sanctuary site and Awash National Park in its territory and hence,

the wild animals (including Oryx, warthog, gazelle and zebra) from the Parks share the majority of the grazing land and watering points in Amibara District. It was very common to observe cattle grazing in close proximity with wild animals in the grazing sites of Amibara District.

3.1.2.2.Dubti District

Dubti district is one the district in Zone One, which is located relatively in the center of the Afar Region approximately at 574 km to the northeast of Addis Ababa in the Lower Awash Valley. Dubti District has 18 *kebeles* (15 rural and 3 urban). The District is bordered on the south by the Somali Region, on the southwest by Mille District of zone 1, on the west by Chifra District of zone 1, on the northwest by Awra District of Zone 4, on the north by Elidaar District, on the east by Aysaita District of Zone 1 and on the southeast by Afambo district. Dubti District has a total human population of 95,886 of which the majority (73%) of the inhabitants are pastoralist whose economy is based on livestock production and few are agro-pastoralist based on mixed farming (i.e., livestock production and small-scale irrigation based crop farming). The Dubti District has one Regional Referral Hospital and two health center. The Dubti Regional Referral Hospital is the only referral hospital in the region and admits all patients (including TB patients) from different districts of the Afar Region and it is the main health facility giving service for region.

The livestock population of the Dubti District is 40,595 (Cattle), 52,727 (goat), (25, 532) sheep, and (29,462) camel (CSA, 2008). The pastoralist communities in the district live in a cluster of 10-15 huts on average in one village.

3.1.2.3.Afambo District

Afambo District is located in the eastern extreme of the Afar Region bordering Djibouti in the east. The district is found in the Lower Awash Basin and an altitude ranging from 280 to 1000masl. The Awash River crosses the district and end in Lake Abe, which found in the district. Numerous smaller lakes, ponds and swampy areas are also found scattered in the eastern parts of Afambo District. Afambo District has seven *kebeles* of which 4 of them are agro-pastoral *kebeles* who mainly producing corn using small-scale irrigation systems from Awash River and the remaining 3 *kebeles* are pastoral. The total human population of

Afambo District is 19, 263 and the livestock population of the district is 28,588 (cattle), 15,592 (goat) (CSA, 2008).

The annual average rainfall in both Dubti and Afambo Districts is around 80 to 216 mm. Three rainy seasons can be identified with varying degree of precipitation – *Karma* (main), *Sugum* and *Dadaa* (the small rains). The average annual temperature ranges between 21^0C and 45^0C. Scattered grassy plains, good for cattle are also found in the two districts. The thick riverine type of vegetation is found along the major streambeds. Animals from Dubti District usually migrate to Chifra District to the west during shortage of pasture for grazing. A large government-owned "Tendaho" Cotton cultivation farm is located in the district and at present there is a wide expansion of this irrigation farm, which has extremely, reducing the grazing land for most of the pastoralists.

3.1.2.4.Chifra District

The district is located 607 Km from Addis Ababa. Chifra is one of the 32 woredas in the ANRS. The district shares boundary with Werebabo district of the Amhara Regional States in the West, on the South by Mile district of zone one, on the North by Ewa and Awra districts of zone four and on the East by Dubti district of zone one of the ANRS. The altitude of the district ranges from 980 to 1000 masl (NMSA, 2000).

The district, with a total area of 12,444 Km^2 (sharing 13.13% of the total area of the region) and divided into 2 agro-climatic zones: arid (Assgura), and semiarid (Mesigidoo), each sharing 72%, and 28% of the total area of the district, respectively. Three rainy seasons were identified with varying degree of precipitation – *Karma* (main from July to September), *Sugum* (from March to April) and *Dadaa* (the small rains from December to January). The altitude of the district ranges from 980 to 1000 meter above sea level (NMSA, 2000). Mile is the main river crossing the district.

The district has 19 sub-districts of which six of them were agro-pastoralists and the remaining were purely pastoralists. The human population of Chifra District is estimated to be 91,078, of which 50,859 (55.84%) were males and 40,219 (44.16%) were females. Majority of the human population in the district live in rural areas, that is 81,946 (89.98%) (CSA, 2007). Ninety percent of the district population is leading a pastoral life by rearing camels, cattle, goats, and sheep. In 2008 the CSA estimated that a total 1,152,678 livestock in the district; of

cattle population 352,346 (representing 0.84% of Ethiopia's total cattle), 342,286 sheep (1.13%), 306,720 goats (3.37%), 126,349 camels (21.85%), and 24,977 donkey (CSA, 2008). The district has one health center in Chifra town and most of TB patients move to neighboring Wollo zone (Dessie and Bati hospitals) of Amhara Region to for diagnosis of tuberculosis.

3.2. Study design and study population

3.2.1. Epidemiology of tuberculosis in livestock

A cross sectional study design was used to carry out the comparative intradermal tuberculin test (CIDT) on cattle, camel, goat and sheep in the four districts of Afar Pastoral Region and sub-districts were included in the study based on the inclusion criteria (accessibility, security, and willingness of the pastoralists to participate in the research). All settlements (villages) in each selected sub-district were included after obtaining the elder's consent to participate in the study. The sample size for epidemiology of TB in cattle and small ruminant was determined according to Thrusfield (1995) considering the recommendation for sample size estimation involving three or more cluster stages (Thrusfield, 1995). Based on this, assuming the expected prevalence of tuberculosis in livestock to be 50% as no previous study was carried out in the region, with a 5%, margin of error and 95% confidence interval the estimated sample size was 1152 (Paper II and III).

Figure 8. Super-herd of cattle in Amibara district, Halidage subdistrict composed of 500-600 cattle

In this study, animals owned by one owner and/or his close relatives, in which the animals shared common grazing sites, watering points, kept at night in common site and move together during migration, were considered as a herd. In settlements, which had super-herd especially in cattle, a larger herd composed 500 to 600 animals (Figure 8); herd selection was made proportionally to represent each cluster in the super-herd.

For the CIDT based TB epidemiology study in livestock of Afar Region a total of 1147 cattle, 2231 small ruminants (1884 goat and 347 sheep) from four districts (Amibara, Dubti, Afambo and Chifra districts) and 324 camels from two districts (Amibara and Chifra districts) above the age of six months and with no clinical symptom of diseases were included in the study. Basic animal information on each tested animals (such as sex, age, breed, body condition score, lactation and reproductive status, parity number (number of calving) were collected and recorded at the time of the test. All the cattle, goat, and sheep included in this study were indigenous local Afar breeds (Paper II and III).

3.2.2. Camel tuberculosis: pathology and molecular characterization of the causative agents

Abattoir-based cross sectional study design was used on 906 apparently healthy camels (male, n =535; female, n= 371) slaughtered at Akaki and Metehara Abattoirs (Paper I). The camels slaughtered were brought to Akaki (Addis Ababa) and Metehara Abattoirs from the two main pastoral regions of Ethiopia, namely Awash-Fentale pastoral area (Kereyu and Afar in the Middle Awash region) and Borena pastoral area (southern Ethiopia). The catchment area in Fentale pastoral area (Middle Awash region) possesses 68,331 camels and in Borena pastoral area of Oromia Regional State, which border with Kenya possesses an estimated population of 97,131 camels (CSA, 2008). In pathological postmortem examination, all camels slaughtered during the study period were included and slaughtered camels were examined for suspected gross TB lesions and tissue lesion samples were collected for isolation and molecular characterization of the causative agents. On average 6–8 camels were slaughtered per day depending on the request from customers. The main consumers of camel meat in Addis Ababa are the Somali immigrants residing in the city.

3.2.3. Small ruminant tuberculosis: Pathology and molecular characterization of the causative agents

For pathology examination of tuberculous lesions in small ruminants and isolation of the causative agents, a cross-sectional study was carried out on a total of 134 goats (129 goats slaughtered at backyard-slaughter sites since there is no abattoir in Afar Region and five strong bovine tuberculin reactor goats which were purchased and slaughtered based on their tuberculin skin test results) were examined for suspected gross pathological lesions and tissue lesions with suspected tuberculous lesions were collected for further isolation and molecular characterization of the causative agents.

3.2.4. Molecular Epidemiology of *Mycobacteria* isolates from human pulmonary tuberculosis patients

Mycobacteria isolates isolated from smear positive sputum samples of human pulmonary tuberculosis patients in Afar Pastoral Region, which were collected during the NUFU-funded

project period (2007-2011), were used to carry out the molecular epidemiology study of tuberculosis in pastoralist communities of Afar Region (**Paper IV**). Hence, 172 *Mycobacteria* isolates were collected from pulmonary TB patients from 11 districts of Afar Pastoral Region who were examined at Dubti Regional Referral Hospital, Awash Health Center, Melaka Werer Health Center and Gewane Heath Centers. Out of these, 62 isolates were collected through community-based active pulmonary TB case-detection study in Amibara District (Legesse *et al*. 2013).

3.3. Body condition scoring and Age determination in livestock

For cattle, the body condition of each of the study animal was scored using the guidelines established by Nicholson and Butterworth (1986). Accordingly, on the basis of observation of anatomical parts such as vertebral column, ribs, and spines, the study animals were classified as lean (score, 1 to 2), medium (3 to 4), or fat (greater than 5).

The body condition scoring for goat and sheep was carried out using the guidelines established by Langston University and ESGIP guidelines for body condition scoring (Langston University, 2007; ESGPIP No.8). Accordingly, on the basis of observation of anatomical parts such as vertebral column, ribs, and spines, the study animals were classified as lean (score 1 to 2), medium (3 to 4), or fat (greater than 5). Age determination was carried out based on the dentition according to Vatta and his colleague guideline (Vatta *et al.,* 2005; ESGPIP No.23).

The condition of a camel is estimated by looking at the store of body fat i.e., in the hump according to the previously established guideline indicated in the website: http://www.camelsaust.com.au/livebodycond.htm ; and the scores range from 1 to 5 based on the amount of fat in the hump which reflects the internal fat reserves and provides a good correlation with total body fat.

3.4. Comparative intradermal tuberculin test in animals

CIDT was carried out by injecting both bovine purified protein derivative (PPD) and avian PPD (Observe™ bovine and avian tuberculin, AsureQuality Company, Mt. Wellington, Auckland, New Zealand). Two sites on the skin of the mid-neck of the animal (cattle, camel,

goat and sheep), 12 cm apart were shaved, and skin fold thickness was measured with a caliper. One site was injected with an aliquot of 0.1 ml of 2,500 IU/ml bovine PPD into the dermis, and the other was similarly injected with 0.1ml of 2,500 IU/ml avian PPD. After 72 h, the skin thickness at the injection sites was measured and recorded. Results were interpreted according to the recommendations of the Office International des Epizooties (OIE, 2009) at ≥ 4 mm cut-off and also at ≥ 2 mm cut-off (Ameni *et al*., 2008). Thus, at cut-off ≥ 4 mm, if the increase in skin thickness at the injection site for bovine PPD (PPD-B) was greater than the increase in skin thickness at the injection site for avian PPD (PPD-A) and PPD-B minus PPD-A was less than 2 mm, between 2 and 4 mm, or 4 mm and above, the animal was classified as negative, doubtful, or positive for BTB, respectively. At cut-off ≥ 2 mm, if the difference between B and A was greater or equal to 2mm, the animal was considered as positive, while if the difference is less than 2 mm, the animal was considered as negative. When the change in skin thickness was greater at PPD-A injection site, the animal was considered positive for mycobacterial species other than MTBC. A herd was considered as positive if it had at least one tuberculin reactor animal.

For camel CIDT test a pilot optimization study were carried out to determine the best skin site of injection (Axilla and middle of the neck) and the time of result reading (at 24h, 48h, 72h and 120h) and based on this the skin on middle of the neck and 72h were the optimum site and time of reading of result of CIDT in camel.

3.5. Postmortem inspection and pathology scoring

Postmortem inspection was performed following the procedure as previously described (Corner, 1994). Mandibular, retropharyngeal, bronchial, mediastinal, mesenteric and hepatic lymph nodes were examined and organs including lungs, liver, small intestine and kidneys were examined in detail during post-mortem in the abattoir under a bright-light source. The lobes of the left and right lungs were inspected and palpated externally. Then, each lobe was sectioned into about 2-cm-thick slices to facilitate the detection of lesions with sterile surgical blades. Similarly, lymph nodes were sliced into thin sections (about 2mm thick) and inspected for the presence of visible lesions. Whenever gross lesions suggestive of TB were detected in any of the tissue, the tissue was classified as having lesions.

Pathology scoring was conducted on tissues with abscesses and tubercle lesions to determine the severity of the lesions based on semi quantitative procedure developed previously

(Vordermeier et al., 2002; Ameni *et al*., 2006). Briefly, lesions in the lobes of the lungs were scored separately as follows: 0 = no visible lesions; 1 = no gross lesions but lesions apparent on slicing of the lobe; 2 = fewer than five gross lesions; 3= more than five gross lesions; 4= gross coalescing lesions. The scores for the individual lobes were summed and generated lung score. Similarly, the severity of gross lesions in individual lymph nodes was scored as follows: 0= no gross lesions; 1= small lesion at one focus; 2 = small lesions at more than one focus; 3= extensive necrosis. Individual lymph node scores were summed and generated the lymph node score. Total pathology score per animal was obtained from the sum of the two total scores.

3.6. Isolation of Mycobacteria

3.6.1. Mycobacterial isolation from animal tissue lesions

For mycobacteriological isolation, tuberculous lesions from slaughtered animals (camel and goat) were aseptically collected into sterile universal bottles with about 5 ml of 0.9% saline solution and kept in icebox with solid packs to keep the cold chain. Then the samples were transported to Aklilu Lemma Institute of Pathobiology (ALIPB) and stored at +4 to $+8^0$C until mycobacteriological culturing was carried out in TB laboratory.

The samples were further processed for isolation of mycobacteria in accordance with the Office International des Epizooties (OIE, 2008; Ameni *et al.*, 2007). The specimens were sectioned using sterile blades, minced with scissors and homogenized with a sterile mortar and pestle under a biological safety cabinet. The homogenates were decontaminated by adding an equal volume of 4% NaOH on the sample in order to remove contaminants. Thereafter, centrifuged at 3,000 rpm for 15 minutes to concentrate the mycobacteria. The supernatant was discarded, and the sediment was neutralized by 1% (0.1 N) HCl acid using phenol red as an indicator. Neutralization was achieved when the color of the solution changed from purple to yellow (OIE, 2008). Next, 0.1 ml of suspension from each sample was spread onto a slant of Löwenstein Jensen (LJ) medium. Duplicate slants were used, one enriched with sodium pyruvate and the other enriched with glycerol. Cultures were incubated aerobically at 37^0C for 8–12 weeks with weekly observation for growth of colonies. Positive cultures were confirmed with Ziehl Nelseen staining and preserved with freezing media while at the same time heat killed in water bath at 80^0C for 45 minutes. The frozen and heat killed isolates were stored at (-20^0C) for further mycobacteriology and molecular typing analysis.

3.6.2. Mycobacterial isolation from milk and nasal swab of tuberculin reactor animals

Milk and nasal swab samples were collected from strong positive tuberculin reactor animals based on CIDT results. Approximately 30 ml of the last streams of milk were collected aseptically into a sterile universal bottle from four quarters of the udder and placed in cool box. Then the samples were transported to Aklilu Lemma Institute of Pathobiology (ALIPB) and stored at +2 to +8^{o}C until mycobacteriological culturing was carried out in TB laboratory.

Nasal swabs and discharges were collected from tuberculin reactor animals aseptically using sterile nasal swab. The nasal swab was inserted into the nose 5-10cm into the nostrils until it reaches the caudal part of the meatus and then the swab was rotated against the nasal mucosa. Using another swab the same procedure repeated in the other nostril. In case of the presence of purulent discharge, the discharge was also aspirated using sterile syringe in addition to the swab. Then the swabs were placed in sterile labeled test tube containing 5ml saline. The sample then transported in cold-chain to ALIPB and stored at +2 to +8^{o}C until mycobacteriological culturing was carried out in TB laboratory.

For isolation of *Mycobacteria* species from milk, the samples were cultured according to Kazwala *et al.* (1998). Briefly, samples were centrifuged at 3000rpm for 15 min and the supernatant discarded. The sediments were suspended in 2 ml of sterile physiological saline solution and decontaminated with equal volume of sterilized 4% NaOH solution. One or two drops of 0.05% phenol red indicator were added and then neutralized using concentrated HCl. The suspension was centrifuged at 3000 rpm for 15 min and the sediment was inoculated onto two slants of Lowenstein-Jensen media. Duplicate slants were used, one enriched with sodium pyruvate and the other enriched with glycerol. Cultures were incubated aerobically at 37°C for about 8- 12 weeks with weekly observation on growth of colonies. Positive cultures were confirmed with Ziehl Nelseen staining and preserved with freezing media while at the same time heat killed in water bath at 80^{o}C for 45 minutes. The frozen and heat killed isolates were stored at (-20^{o}C) for further mycobacteriology and molecular typing analysis. A similar procedure was followed for culturing nasal swab samples.

3.6.3. Mycobacterial isolation from human sputum samples

Acid-fast bacilli (AFB) smear positive sputum samples obtained from individual PTB patients from each health facilities in Afar Region, upon reaching the ALIPB TB laboratory, specimens were decontaminated for 15 minutes with an equal volume of 4% NaOH and centrifuged at 3000 revolution per minute (rpm) for 15 minutes. The supernatant was decanted while the sediment was neutralized with 1% (0.1N) HCl using phenol red as an indicator. Neutralization was achieved when the colour of the solution was changed from purple to yellow. Then, 0.1 ml of the pellet was inoculated onto Lowenstein-Jensen medium containing pyruvate or glycerol and incubated for 10 weeks at 37^0C. Cultures were followed weekly for the growth of mycobacterial colonies and confirmed by AFB microscopy. AFB-positive isolates were prepared by mixing two loops full of colonies in 200 ml distilled water, heat-killed at 80^0C for 1 hour using water bath, and stored at -20^0C until molecular characterization was performed.

3.7. Molecular characterization of mycobacterial isolates

3.7.1. Multiplex polymerase chain reaction (mPCR)

Multiplex polymerase chain reaction (Mycobacterial genus typing) was conducted as described previously (Wilton and Cousins, 1992). Heat killed mycobacterial isolates were used as source of DNA template and amplifications was done in thermocycler (VWR Thermocycler, 732-1200, Leicestershire, UK) with 20 µl reaction volumes consisting: 5 µl of genomic DNA template, 8 µl HotstarTaqMasterMix (MgCl2, dNTP, Taq polymerase and PCR buffer) for each sample, 0.3 µl forward and reverse primer per each sample and 5.2 µl per sample of Qiagen water. The PCR targets the sequence of the Genus Mycobacterium within the 16S rRNA gene (G1, G2), sequences within the hyper-variable region of 16S rRNA that is known to be specific to *M. intracellularae* (MYCINT-F) and *M. avium* (MYCAV-R), and the MTB70 gene specific for *M. tuberculosis* complex (TB-1A, TB-1B). The primers used for amplification were MYCGEN-F, 5`AGA GTT TGA TCC TGG CTC AG 3` (35ng/µl); MYCGEN-R, 5`TGC ACA CAG GCC ACA AGG GA 3` (35ng/µl); MYCAV-R, 5` ACC AGA AGA CAT GCG TCT TG 3`(35ng/µl); MYCINT-F, 5`CCT TTA GGC GCA TGA TGT CTT TA 3`(75ng/µl); TB1-F, 5` GAA CAA TCC GGA GTT GAC AA 3` (20ng/µl); TB-1-R, 5` AGC ACG CTG TCA ATC ATG TA 3` (20ng/µl).

M. tuberculosis strains (H37Rv) and *M. avium* were used as positive control while Qiagen water was used as negative control. The reaction mixture was then be heated in thermocycler using the following amplification program: 95^0C for 10 minutes for enzyme activation; 95^oC for 1 minute for denaturation; 61^0C for 0.5 minute for annealing; 72^0C for 2 minutes for extension, involving 35 cycles all in all; and final extension at 72^0C for 10 minutes.

The product was electrophoresed in 1.5% agarose gel in 10x TAE running buffer. Ethidium bromide at ratio of 1:10, 100bp DNA ladder, and orange loading dye (6X) were used in gel electrophoresis. Interpretation of the result was made following the Standard operating procedure (SOP) developed by the Animal Health and Veterinary Laboratories Agency, UK (AHVLA, 2009), which states that all members of the Genus *Mycobacterium* produce a band of 1030bp, *M. intracellularae* a band of 850bp, members of *M. tuberculosis* complex produce a band with 372bp while *M. avium* subspecies *avium* or subspecies such as *M. avium* subspecies *paratuberculosis* produces a band of 180bp.

3.7.2. Region of difference (RD)-4 deletion typing

PCR analysis based on RD regions has been found to be an important differentiating tool between members of the *M. tuberculosis* complex. RD4 is 12.7 kb genetic segment that is deleted from *M. bovis* BCG strain, but present in *M. microti*, *M. africanum*, and *M. tuberculosis* (Gordon *et al.* 1999).

The RD4 deletion typing was carried out on isolates that showed band for *M. tuberculosis* complex by multiplex PCR. For this deletion typing, the procedure described by Cadmus and coauthors was followed (Cadmus *et al.*, 2006). Each sample was tested in a separate PCR tube. Primers directed against the RD4 were used to generate a deletion profile that would allow species identification of the isolate. Primers that were used include RD4intF ACA CGC TGG CGA AGT ATA GC, RD4flankF CTC GTC GAA GGC CAC TAA AG and RD4flankR AAG GCG AAC AGA TTC AGC AT to check for the presence of RD4 locus. The HotStarTaq Master Mix system from Qiagen was used for PCR, with primers described previously. The reaction mixture was 10 ml of HotStarTaq Master Mix, 0.3 ml63 of each primer (flank R, F and int), 2 ml DNA template and 7 ml distilled water to a final volume of 20 ml. M. tuberculosis H37Rv and M. bovis 2122/97 were used as positive control while

Qiagen water was used as negative control. The mixture was heated in Programme Thermal Controller (Applied biosystem; PTC- 100TM) using an initial hot start of 95^0C for 15 minutes, followed by 35 cycles of 95^0C for 1 minute, 55^0C for 1 minute, and 72^0C for 1 minute; a final extension step of 72^0C for 10 minutes to complete the cycle. PCR products were electrophoresed in 1% agarose gel in 16 TAE running buffer, Ethidium bromide at ratio of 1: 10, 100bp DNA ladder and orange 66loading dye were used in electrophoresis. The gel was visualized in Multi–imageTM light cabinet using Alpha Innotech version 1.2.0.1(Alpha Innotech Corporation). The presence of RD4 (*M. tuberculosis*, *M. africanum*) gives a product size of 335bp (RD4int+RD4FlankR) and its absence (*M. bovis*) gives a product size of 446bp (RD4FlankR+RD4FlankF).

3.7.3. Spoligotyping

The spoligotyping was done at Aklilu Lemma Institute of Pathobiology Addis Ababa University (Ethiopia) and at National Reference Laboratory for Mycobacteria in The Norwegian Institute Public Health (Oslo, Norway). Spoligotyping was performed as previously described by Kamerbeek and coauthors (Kamerbeek *et al.*, 1997) and according to the spoligotype kit supplier's instructions (Ocimum Biosolutions Company, Iisselstein, The Netherlands). The direct repeat (DR) region was amplified by PCR using oligonucleotide primers derived from the DR sequence. A total volume of 25 ml the following reaction mixture was used for the PCR: 12.5 ml of HotStarTaq Master Mix (Qiagen: this solution provides a final concentration of 1.5 mM MgCl2 and 200mM of each deoxynucleotides triphosphates), 2 ml of each primer (20 pmol each), 5 ml suspension of heat-killed cells (approximately 10 to 50ng), and 3.5 ml distilled water. The mixture was heated for 15 minutes at 96^0C and then subjected to 30 cycles of 1 minute at 96^0C, 1 minute at 55^0C, and 30 seconds at 72^0C. At the end, the reaction mixture was maintained at 72^0C for 10 minutes. Then amplified product was mixed with 150µl 2x SSPE (16SSPE is 0.18 M NaCl, 10 mM NaH2PO4, and 1 mM EDTA[pH 7.7])-0.5% sodium dodecyl sulfate buffer and the mixture was denatured at 99^0C for 10 minutes and immediately put on ice. Then the product was hybridized to a set of 43 immobilized oligonucleotides, each corresponding to one of the unique spacer DNA sequences within the DR locus. After hybridization, the membrane was washed twice for 10 minutes in 2x SSPE-0.5% sodium dodecyl sulfate at 60^0C and then

incubated in 1:4000 diluted streptavidin peroxidase (Boehringer) for 45 to 60 minutes at 42^0C. The membrane was washed twice for 10 minutes in 2x SSPE-0.5% sodium dodecyl sulfate at 42^0C and rinsed with 2x SSPE for 5 minutes at room temperature. Hybridizing DNA was detected by the enhanced chemiluminescence method and images were captured by exposure to X-ray film (Hyperfilm ECL, Amersham) as specified by the manufacturer at Aklilu Lemma Institute of Pathobiology Addis Ababa University (Ethiopia) or using BioRAD Molecular Imager®. ChemiDoc™ XRS+ Systems with Image Lab™ Software 3.0.1. (Beta) (Bio-Rad Laboratories, Inc., USA) as specified by the manufacturer at National Reference Laboratory for Mycobacteria in The Norwegian Institute Public Health (Oslo, Norway). .

3.7.4. MIRU-VNTR 24- loci genotyping

MIRU-VNTR typing was performed as described previously by Supply *et al.,* 2006. In brief, heat-killed samples were amplified using multiplex PCR of a panel of 24-MIRU-VNTR loci primers and conditions for their amplification as described in the standard protocol (Supply *et al.*, 2006). DNA fragments were separated by capillary electrophoresis using automated ABI-3730 DNA analyzer with 48 capillaries (Applied Biosystems, Foster City, CA). Sizing of the PCR fragments and assignment of the various MIRU-VNTR alleles were done by using the GeneScan LIZ1200 Size Standard and customized GeneMapper software package, Version 3.7(Applied Biosystems). MIRU-VNTR typing was carried out at the Norwegian Institute of Public Health (Oslo, Norway) in The National Reference Laboratory for an accredited laboratory by the European Centre for Disease Prevention and Control and Dutch National Institute for Public Health and the Environment for 24 locus MIRU-VNTR typing. *M. tuberculosis* strain lineages, clustering and phylogenic similarity were analyzed using on-line databases, the MIRU-VNTR*plus* database http://www.miru-vntr*plus*.org/ (Allix-Béguec *et al.,* 2008; Weniger *et al.*, 2010) and for TB-lineage and families using SPOTCLUST database, http://tbinsight.cs.rpi.edu/about_spotclust.html. Clusters were defined as group of MTBC strains with 100% identical MIRU-VNTR and spoligotype patterns.

3.7.5. GenoType® Mycobacterium CM/AS

GenoType® Mycobacterium CM/AS, Common Mycobacterium/Additional Species, (HAIN LIFE SCIENCE) was used for the characterization of the non-mycobacterium tuberculosis members. The test is based on the DNA STRIP® technology. The PCR reaction consisted of 35µl PNM, 5µl 10x PCR buffer, 2µl 25mM $MgCl_2$ solution, 0.2µl (1U) HotStarTaq, 3µl of qiagen water and 5µl of DNA template and the PCR condition was set at heating at 95^0C for 5 min, and 10 cycles of denaturation at 95^0C for 30 sec and annealing at 58 ^{0}C for 2 min. This followed by 20 cycles consisting of denaturation at 95^0C for 25 sec, annealing at 53 ^{0}C for 40 sec, and extension at 70 ^{0}C for 40 sec and finally one cycle of extension at 70^0C for 8 min. For running the test, the procedure described in the manual enclosed in the test kit (Hain life science GmbH, Hardwiesenstraβe 1, 72147 Nehren, Germany, www.lifescience.de) was followed. Briefly, TwinCubator® (water bath), hybridization buffer, and stringent wash solution were pre-warmed at 45oC. A tray with 12 wells, provided with the kit was used for the test. Conjugate concentrate and conjugate diluents were mixed in a ratio of 1:100. Substrate concentrate and substrate diluents were also mixed in a similar concentration. Twenty micro liter of denaturation solution was added in the corner of the wells and thereafter 20µl of amplified sample was added to the wells and mixed and incubated at room temperature for 5 min. One ml of pre-warmed hybridization buffer was added into the well and mixed until the solution became homogenous. Strips were placed in each well and the tray was placed in a shaking TwinCubator® and incubated for 30 min at 45oC. Then, the hybridization buffer was aspirated and 1 ml of Stringent Wash Solution was added into each well and incubated for in shaking TwinCubator® for 5 min at 45oC. The Stringent Wash Solution was completely removed, and each well was washed with 1 ml of Rinse Solution for 1 min in shaking TwinCubator® at room temperature. One ml of diluted conjugate was added to each well and incubated for 30 min at room temperature in a shaking TwinCubator®. The solution was removed and each well was washed twice with 1 ml of Rinse Solution in a shaking TwinCubator® for 1 min, and once in 1 ml of distilled water. Then, 1 ml of diluted substrate was added into each well and incubated at room temperature in dark condition for 10-15 min. Finally, the strips were removed using tweezers and placed between two layers of absorbent paper. The result was interpreted based on the bands following the interpretation key supplied with the kit.

3.7.6. 16S rDNA sequencing

Of the 19 mycobacterial isolates from camel tuberculosis study which were characterized as non-tuberculosis mycobacteria using multiplex PCR 18 isolates were subjected to 16S rDNA sequencing and for each sample ~600bp of the 16S rDNA gene has been sequenced at hyper variable region A and hyper variable region B. DNA sequencing was performed by The Sequencing Service at Animal Health and Veterinary Laboratories Agency (AHVLA, Weybrige, UK) using Applied Biosystems Big-Dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer. BLAST searches to find homology to known species/sequences were done at RIDOM and NCBI databases, websites below: http://rdna2.ridom.de/ridom2/ and http://blast.ncbi.nlm.nih.gov/ .

3.8. Ethical considerations

The study protocol was approved by the Ethical Clearance Committee of the Aklilu Lemma Institute of Pathobiology (ALIPB) (Reference no. IPB-RPC-01/07), Addis Ababa University (Ethiopia), and by the Regional Committee for Medical Research Ethics of Southern Norway since the work was a collaborative research with University of Oslo (Norway). Verbal consent was obtained from their owners for all animal studies and written consent was obtained from pulmonary tuberculosis patients who involved in the human molecular epidemiology study.

3.9. Data management and analysis

Data were classified, filtered, coded using Epidata software and Microsoft Excel sheet, and was transferred and analyzed using STATA version 11 (Stata Corp., Collage station, TX). Pearson chi- square was used to evaluate the statistical significance of the associations of different categorical variables with skin test results and McNemar's chi-square was used to assess the association of PPD-A and PPD-B results. Bivariate and multivariable logistic regression analyses were performed to quantify crude and adjusted effects of pre-specified

risk factors on tuberculin reactivity. p-value less than 5% was considered statistically significant. In cases of estimating the effect of different risk factors in terms of odds ratio (OR) with corresponding 95% confidence interval, statistical significance was assumed if the confidence interval did not include one among its values.

In camel pathology study, mean and standard error of mean were used to summarize pathology scores and Friedman test was used to compare pathology score of tropism of TB lesions among lymph nodes as well as among lung lobes.

In molecular epidemiology study of isolates from human pulmonary tuberculosis patients, the spoligotype patterns were converted in to binary and octal formats and entered to the on-line spoligotype database, http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/index.jsp to determine the shared international spoligotype (SIT) number and the results were compared with already existing designations in the international spoligotyping database (SpolDB4.0 database). Those isolates with no designated SIT number were considered as new to the database. Two or more isolates with identical spoligotype pattern were considered as clustered while those with single SIT were considered as non-clustered isolates. Using the MIRU-VNTR*plus* database http://www.miru-vntr*plus*.org/ the MIRU-VNTR 24-loci profile and spoligotype patterns were used to designate the strains MLVA MtbC15-9 type, SIT numbers and phylogenic lineages as compared to the reference strains in the database. In addition, cluster analyses and dendogram was generated by the online MIRU-VNTR*plus* database software using combined data of the spoligotype pattern and MIRU-VNTR data. TB-lineage and family were determined using SPOTCLUST database, http://tbinsight.cs.rpi.edu/about_spotclust.html.

4. RESULTS

4.1. Camel tuberculosis: Epidemiology, Pathology and molecular characterization of its etiology

4.1.1. Epidemiology of camel tuberculosis

Comparative intradermal tuberculin (CIDT) was carried out on 324 camels from two districts (Amibara and Chifra districts), of which the final analysis was carried out on 307 camels. Based on CIDT test result, the overall individual animal prevalence of camel TB in the two districts of Afar was 16.6% [95% CI: 12.4%-20.8%] at a ≥4mm cut-off point and 20.8% [95% CI: 16.3%-25.4%] at a ≥2mm cut-off point (Figure 9).

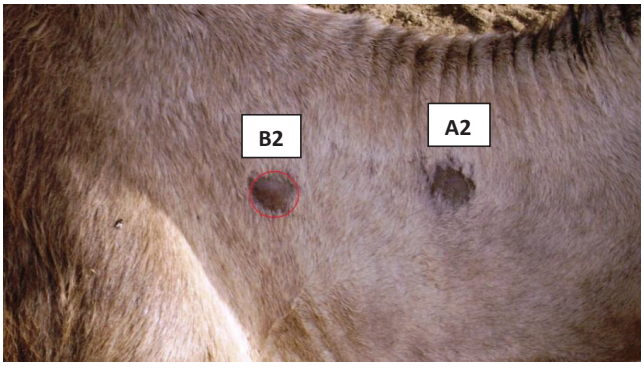

Figure 9. Bovine tuberculin reactor camel showing induartion (B2) at bovine tuberculin PPD injected site on the neck while no swelling at avian tuberculin injected site (A2)

At ≥4mm cut-off point, there was significant difference in proportions of camel bovine tuberculin reactors among the three age categories ($\chi 2 = 7.7279$, $P = 0.021$), the highest prevalence was in the older camels above the age of 8years (21.6%) while the lowest prevalence was in younger camels below the age of 4years (5.9%) (Table3). At ≥4mm cut-off point, there was no statistical significance difference in the proportion of bovine tuberculin positivity between camel groups in relation to other variables considered

(districts, sex, body condition score, lactation status, and reproductive status) (Table3). According to the CIDT test result of the avian tuberculin skin reaction, the overall animal proportion of avian tuberculin reactors was 23.1% (95% CI: 18.7%–27.95%) at ≥4mm cut-off point. A statistically significant difference in proportion of avian tuberculin reactor camels between districts ($\chi 2 = 5.07$, $P = 0.02$) in which the highest proportion was in Amibara District (24.9%), while the lowest proportion was in Chifra District (6.7%). Similarly, between female and male camels, there was a statistically significant difference in avian tuberculin reactivity were seen in which female camel had the highest proportion (24.6%) while male camel showed the lowest proportion (7.7%) in avian tuberculin reactivity.

Table 3: Association of different risk factors to skin test positivity at 4mm cut-off points for camel tuberculosis in Afar Pastoral Region of Ethiopia

Variable	Number of camel tested	Number of positive (%)	Chi-square	p-value
Districts				
Amibara	277	49 (17.7)	2.374	0.123
Chifra	30	2 (6.7)		
Sex				
Female	281	49 (17.4)	1.632	0.201
Male	26	2 (7.7)		
Breed				
Afar	291	63 (21.7)	2.1796	0.140
Somali	16	1 (6.3)		
BCS				
Poor	110	17 (15.5)	0.4947	0.781
Medium	163	27 (16.7)		
Good	34	7 (20.6)		
Age (years)				
<4	51	3 (5.9)	7.7279	0.021*
4 ≤ X < 8	88	12 (13.6)		
≥ 8	167	36 (21.6)		
Lactation status				
Lactating	68	12 (17.7)	0.1245	0.724
Non-Lactating	29	6 (20.7)		
Reproductive status				
Pregnant	13	2 (15.4)	0.0277	0.868
Non-pregnant	66	9 (13.6)		

BCS: body condition score ; *statistically significant

Multivariable logistic regression analysis at ≥4mm cut-off point showed that older camels (8 years and above) had 4 times the odds of being tuberculin reactors compared with the younger age group (less than 4 years old) (adjusted OR=4.27; 95% CI: 1.23-14.84) which is

statistically significant (Table 4). In relation to other factors considered, there were no statistical significance differences of bovine tuberculin positivity between groups.

Table 4: Multivariable logistic regression analysis of bovine tuberculin reactors with associated risk factors at 4mm cut-off point in camels of Afar Region

Variable	Number of camel tested	Number of positive (%)	Crude Odd ratio (95% CI)	Adjusted Odd ratio (95% CI)
Districts				
Amibara	277	49 (17.7)	1	1
Chifra	30	2 (6.7)	0.33(0.08-1.44)	0.32(0.07-1.46)
Sex				
Female	281	49 (17.4)	1	1
Male	26	2 (7.7)	0.39(0.09-1.72)	0.59 (0.12-2.81)
Age (years)				
<4	51	3 (5.9)	1	1
4 ≤ X < 8	88	12 (13.6)	2.53(0.68-9.42)	2.64 (0.69-10.15)
≥ 8	167	36 (21.6)	4.40(1.29-14.94) *	4.27(1.23-14.84)*
BCS				
Poor	110	17 (15.5)	1	1
Medium	163	27 (16.7)	1.09(0.56-2.10)	0.92 (0.46-1.84)
Good	34	7 (20.6)	1.42(0.53-3.78)	1.37 (0.49-3.82)

BCS: body condition score ; *statistically significant

With respect to the result of abattoir-based epidemiology of camel tuberculosis, out of 906 slaughtered camels inspected for the presence of gross tuberculous lesions in different organs 91 camel had gross pathology for tuberculous lesion. Hence, the prevalence of camel tuberculosis in Ethiopia was 10% (91/906). Culture positivity was confirmed in 34% (31/91) of the camels with suspicious TB lesions. The result of the association of the different risk factors to the pathology showed that having a good body condition has a protective effect against being positive for TB (shown in Table 1 of Paper I).

4.1.2. Pathology of camel tuberculosis

The distribution of lesions and the severity of the disease were established in the 91 camels with suspicious lesions. The tropism of TB lesions to specific lymph nodes and lung lobes was statistically significant among the lymph nodes (χ^2 = 22.697; P= 0.002) and lung lobes (χ^2 = 17.901; P = 0.006) (Table 2). Lung lesions were detected in 43 camels while 78 camels had at least one lesion in their lymph nodes. The lesions appeared more frequent in the apical

and cardiac lobes of both lungs than in the diaphragmatic lobes (Table 2). Similarly, the severity was greater in both right apical and cardiac lobes. Regarding lymph nodes, mesenteric lymph nodes were found the most frequently and severely affected of all the lymph nodes (34%) (Table 2 and Figure 1 of Paper I and Figure 10).

Figure 10. Disseminated tuberculous lesions on the small intestine and mesenteric lymph nodes

The mean severity of pathology of camel TB is summarized in Table 3 of Paper I. The mesenteric lymph node constituting the most severely affected lymph node followed by mediastinal lymph node (0.64±0.11; 0.55±0.15) respectively, and the parotid lymph node is the least affected (0.27±0.08). One of the camels had a generalized disseminated TB in which the tuberculous lesions were observed in different organs and lymph nodes of the camel.

4.1.3. Isolation and Molecular characterization of Mycobacterial isolates from camel tuberculous lesions

Growth of mycobacteria was observed in 34% (31/91) of camels with suspicious TB lesion (Figure 2 on Paper I). Culture positivity was highest (58.8%) in the retropharyngeal lymph node followed by the mesenteric lymph node (35.5%). In contrast, mycobacterial culture positivity from mandibular and parotid lymph nodes was less frequently with 13.3% and 15.4%, respectively.

Further Mycobacterium genus typing was conducted using multiplex PCR on the 31 culture isolates from camels. Based on the result, 21 isolates gave signal to the genus M*ycobacterium*. Two of these isolates were confirmed to be members of the *M. tuberculosis* complex (Figure 3 of Paper I). The two isolates that showed signal to *M. tuberculosis* complex were subjected to RD4 deletion typing and they were confirmed to be *M. bovis* (Figure 4 of Paper I or Figure 11 on this thesis).

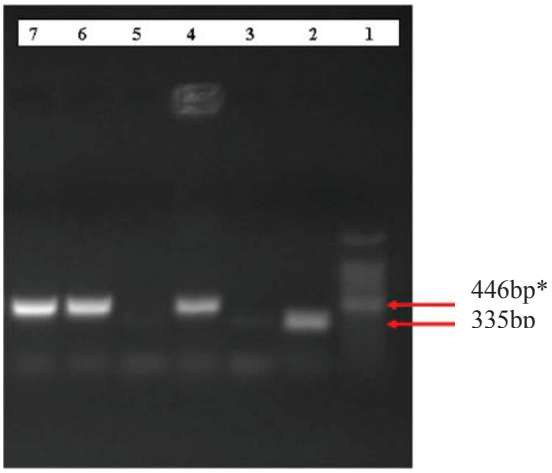

Figure 11. Gel electrophoresis showing the PCR products of the two *M. bovis* isolated from camel tuberculous lesion by RD4 deletion typing. Lane 1=100bp DNA ladder, Lane 2=*M. tuberculosis* (positive control), Lane 3=Qiagen H_2O (negative control), Lane 4= *M. bovis* (positive control), Lane 5-7 were isolates from camel, Lane 6 and 7 were positive for *M. bovis*. ** Correction has been made on the position of the labeling of the band size in Paper I*.

Spoligotyping of the two isolates of *M. bovis* confirmed that one of the isolate was SB0133 strain and the other one was a new strain, which was not reported previously in www.mbovis.org database. The new strain was reported to the global database

(http://www.mbovis.org) and designated as SB1953 (Figure 5 of Paper I). The SB0133 isolate was isolated from camel with generalized and disseminated form of TB.

Further molecular characterization of 18 of non-tuberculous mycobacteria isolated from camel tuberculous lesions using 16S rDNA sequencing at Animal Health and Veterinary Laboratories Agency (AHVLA), Weybridges (UK) revealed that 77% (14/18) were members *Mycobacterium terrae* complex. Other species identified include *M. flavescens, M. acapulcensis, M. chelonae, M. moriokaense,* and *M. avium.*

4.2. Small ruminant tuberculosis: Epidemiology, Pathology and Molecular characterization of its etiology

4.2.1. Epidemiology of tuberculosis in small ruminants of Afar Region

Comparative intradermal tuberculin (CIDT) was carried out on a total of 2231 small ruminants (1884 goat and 347 sheep) and on the basis of this test result, the overall individual animal prevalence of TB in small ruminants was 0.5% (10/2231) at a cutoff ≥4mm and 3.8% (86/2231) at a cutoff ≥ 2 mm.

At ≥2mm cut-off point, there were significant differences in proportions of reactors among the four districts ($\chi 2 = 26.385$, $P = 0.000$) the highest prevalence being in Dubti district (9.28%) while the lowest prevalence was in Amibara district (2.63%). Similarly at ≥2mm cut-off point a statistically significant difference were observed in prevalence of bovine tuberculosis between sheep (1.44%) and goat (4.3%) ($\chi 2 = 6.46$, $P = 0.011$) and between pregnant and non-pregnant females ($\chi 2 = 5.342$, $P = 0.021$) (Table 1 of Paper II). Multivariable logistic regression analysis at ≥2mm cut-off point showed that older small ruminants (5 years and above) had 13 times the odds of being tuberculin reactors compared with age category less than 2 years old (adjusted OR = 13.79; CI: 2.22–85.55). Female small ruminants with parity number greater than 4 had 0.05 odds of being bovine tuberculin positivity in relative to those with less than 2 parity numbers (adjusted OR = 0.05; CI: 0.01–0.31) (Table2 in Paper II). At ≥4mm cut-off points, there was no statistical significance difference in the proportion of bovine tuberculin positivity between groups in relation to the different variables considered.

The herd prevalence was 20% (95% CI: 12–28%) and 47% (95% CI: 37–56%) at ≥4mm and ≥2mm cut-off points, respectively. In multivariable logistic regression analysis, no significant association was found in herd positivity between groups in relation to district of origin, herd size category, and production system at ≥2mm cut-off point (Table 3 in Paper II).
According to the CIDT test result of the avian tuberculin skin reaction, the overall animal prevalence of *Mycobacterium avium* complex infection was 2.8% (95% CI: 2.1–3.5%) and 6.8% (95% CI: 5.8–7.9%) at ≥4mm and ≥2mm cut-off points, respectively.

4.2.2. Pathology of tuberculosis in goat

On the basis of detailed inspection of 129 goats slaughtered at backyard slaughtering site in Dubti district the presence of suspicious gross tuberculous lesions were detected in 7(5.4%) goats and the lesions were observed in the lung, retropharyngeal, cranial mediastinal lymph node and mesenteric lymph nodes. On the other hand, five goats, which were strong positive reaction at bovine tuberculin PPD in CIDT (Figure 11) test, were purchased, slaughtered, and investigated for gross tuberculous lesions. Tuberculous lesions were detected in different organs (diaphragmatic lobe of lung, retropharyngeal lymph node, parotid lymph node, right bronchial lymph node, mesenteric lymph node, intestinal wall, and mesentery) (Figure 11 and 12). Two of them had partially disseminated TB lesions, which involved lung, intestine and the lymph nodes of thoracic and abdominal cavities. Upon incision of the lung, lesions showed a yellowish caseous material indicating a characteristic of tuberculous lesion (Figure 1 of Paper II). In one goat both avian and bovine tuberculin injection site showed swelling and up on postmortem examination a generalized distribution of tuberculous lesions were observed on different organ including intestine, mesentery and mesenteric lymph nodes, yellowish to greenish caseous lesions were observed in the lesions (Figure 12).

Figure 12. Strong bovine tuberculin reactor goat (induartion of 11mm) (1) and tuberculous lesion obtained on the lung from the reactor goat after slaughtering indicated by arrow (2) B2: indurations of skin at bovine tuberculin injection site after 72 h and A2: indurations of skin at avian tuberculin injection site after 72 h.

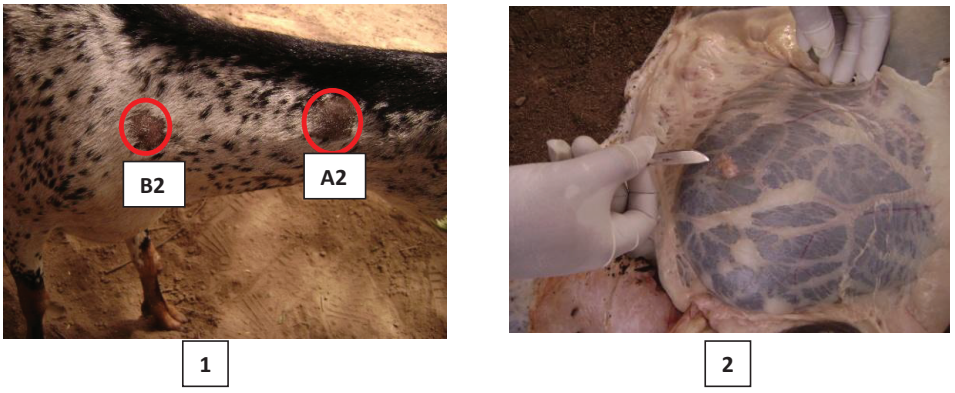

Figure 13. Strong avian and bovine tuberculin reactor goat (1) and tuberculous lesion obtained on mesentery from the reactor goat after slaughtering indicated by scalpel tip (2) B2: indurations of skin at bovine tuberculin injection site after 72 h and A2: indurations of skin at avian tuberculin injection site after 72 h.

4.2.3. Isolation and Molecular characterization of mycobacterial isolates from goats

Mycobacterial cultures of all the five suspicious tuberculous lesions obtained from slaughtered tuberculin reactor goats were positive for mycobacterial growth on LJ culture medium. Further molecular characterization indicated that one of the isolates was human type *Mycobacterium tuberculosis* (SIT149) from goat specimen (Figure 2 of Paper II), and the others were non-tuberculosis mycobacteria species. The goat with SIT149 isolate was strong reactor to bovine tuberculin test with high skin induration difference (PPD-B minus PPD-A = 10 mm), and the postmortem examination result showed typical tuberculin lesions in lung (Figure 12), bronchial lymph nodes, caudal mediastinal lymph node, and also on mesenteric lymph nodes while goats from which non-tuberculosis mycobacteria species were isolated have showed indurations of skin at both avian and bovine tuberculin injection site. In addition, the pathological lesions observed in postmortem examination were localized in retropharyngeal lymph nodes, mesentery, intestine and mesenteric lymph nodes (Figure 13).
From the seven tuberculous lesion collected from postmortem examination of 129 slaughtered goats mycobacterial growth were detected in three of the samples and molecular characterizations using multiplex PCR of these one isolate showed band signal for genus *Mycobacteria* and the rest were not belong to mycobacterium species.

4.3. Bovine tuberculosis in cattle

4.3.1. Epidemiology of BTB in cattle

Based on CIDT, the animal prevalence of BTB was 11% (119/1087) with 4 mm cut-off point and 18.4% (200/1087) with 2 mm cut-off point (Figure 14). At 4 mm cut-off point, there were statistically significant differences in proportions of bovine positive reactor animals between the four districts ($\chi2 = 21.7$, $P = 0.000$), herd size category ($\chi2 = 8.72$, $P = 0.013$), sex ($\chi2 = 6.96$, $P = 0.008$), age category ($\chi2 = 21.12$, $P = 0.000$) (Table 1 in Paper III). At 2 mm cut-off point, in addition to the factors indicted above, there was a statistically significant difference in proportion of bovine positive reactors between the pastoral and agro-pastoral production system ($\chi2 = 3.8$, $P = 0.05$) where a higher proportion of positive reactors in cattle under pastoral production system than those in agro-pastoral production system. Multivariable logistic regression analysis (Table 2 in Paper III) showed that older cattle (9

years and above) had 2.66 times the odds of being tuberculin reactors compared with those cattle less than 2 years old (adjusted OR = 2.66; CI =1.21-5.84). Cattle found in Amibara district had also the higher odds of being tuberculin positivity in relative to those cattle in Chifra district (adjusted OR = 6.56; CI = 1.63 to 28.73). At both cut-off points, there was no statistical significance difference in the proportion of bovine tuberculin positivity between groups in relation to body condition score, breed, gastrointestinal parasite infestation status, lactation status, reproductive status, and number of parity. The gastrointestinal parasite infestation status in general was low both in tuberculin nonreactors and reactor cattle. In majority of the tested animals, eggs of *Trichostronglus* species were the most common parasite eggs identified in this study.

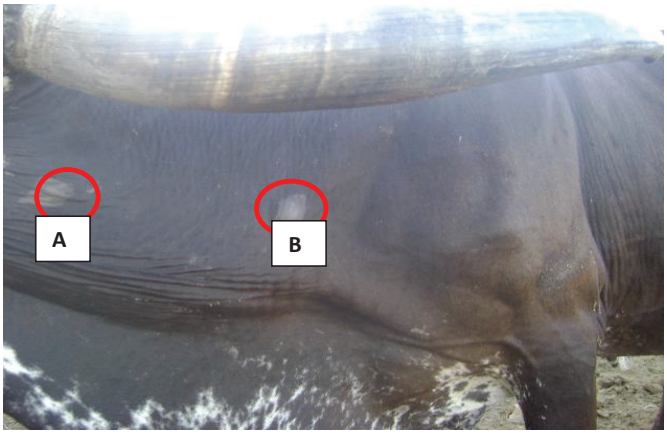

Figure14. Strong bovine tuberculin reactor cattle after comparative intradermal tuberculin test, B2: indurations of skin at bovine tuberculin injection site after 72h and A2: indurations of skin at avian tuberculin injection site after 72h.

The herd prevalence was 44% (95% CI = 36 to 51%) and 56% (95% CI = 48 to 63%) at ≥ 4 mm and ≥ 2 mm cut-off points, respectively. In multivariable logistic regression analysis, herds found in Amibara district had the higher odds of showing tuberculin positivity in relation to those cattle in Chifra district (adjusted OR = 8.15; 95% CI = 1.77 to 37.59), and no significant association was found between herd positivity, herd size and production system (Table 3 in Paper III).

4.3.2. Molecular characterization of isolates from milk and nasal swab of tuberculin reactor animals

Out of the total samples (90 nasal swab, 55 milk and 5 skin nodule/pus) cultured on LJ medium, 30 (25 nasal swab, 4 from milk and 1 from pus of skin nodule) were positive for mycobacterial growth. *Mycobacterium* genus typing using mPCR and Genotyping HAIN analysis were conducted on these 30 mycobacterial isolates. Based on multiplex PCR genus typing, 13 isolates (2 milk samples and 11 nasal swabs and 1 skin nodule samples) were members of non-tuberculosis and Genotyping HAIN analysis identified *Mycobacteria* species (*M. pheli, M. simiae, M. fortitum)* (Figures 15-16 and Figure 18).

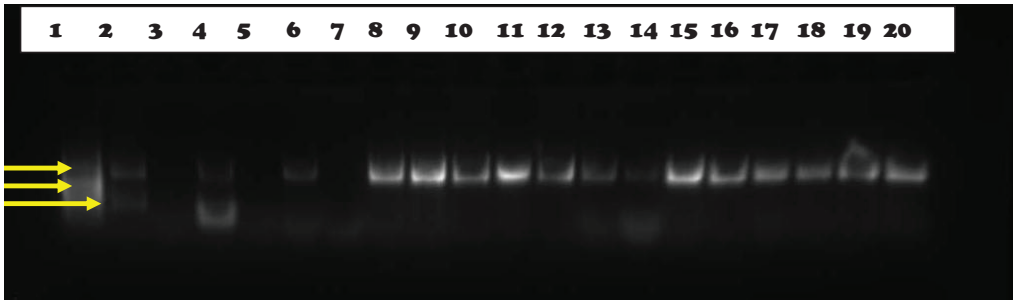

Figure 15. **G**el electrophoresis separation of PCR products by multiplex PCR genus typing of mycobacteria isolated from tuberculin reactor animals in Afar (Gel 1). Lane 1= Ladder 100bp, Lane 2= *Mtb* control, Lane 3=H_2O, Lane 4= *M. avium* control, Lane 5= dt 2 Dubti bovine; Lane 6=DL01G skin nodular lesion bovine; Lane 7=DL30G pus bovine; Lane 13= 7 P nasal swab bovine; Lane 14=3G Andido nasal swab bovine. Lane 6, 13, 14 were positive while Lane 5 and 7 were negative. Lane 8-12, 15-20 was samples from nasal swab of goats and camels.

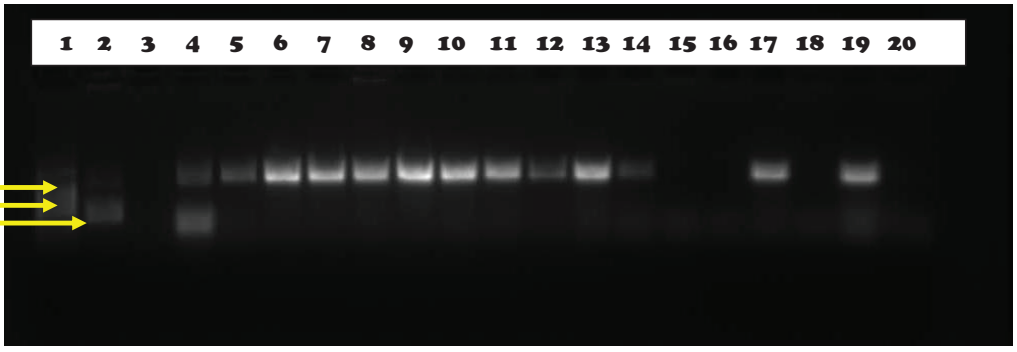

Figure 16: Gel electrophoresis separation of PCR products by multiplex PCR genus typing of mycobacteria isolated from tuberculin reactor animals in Afar (Gel 2). Lane 1= Ladder 100bp, Lane 2= *Mtb* control, Lane 3=H_2O, Lane 4= *M. avium* control, Lane 10= 9G nasal swab bovine, Lane 11=13 P nasal swab bovine, Lane 12 = 5P Gontibirka bovine, Lane 14 =5G Gontibirka bovine, Lane 17= DL31G bovine nasal swab. Lane 10-12, 14, 17 were positive. Lane 5-9, 13, 15, 16, 18-20 were isolates from nasal swab of goats and camels.

4.4. Molecular epidemiology of *Mycobacteria* isolates from human pulmonary tuberculosis patients of Afar Region

4.4.1. Genetic and geographic distribution of *Mycobacteria* isolates from pulmonary tuberculosis patients

Out of the total 172 isolates recovered from pulmonary TB patients, 150 isolates (87.2%) were MTBC and 22 (12.8%) were non-tuberculosis mycobacteria species. Of the 150 MTBC, 148 (98.7%) were *M. tuberculosis* (Figure 17) and 2 (1.3%) were confirmed to be *M. bovis*.

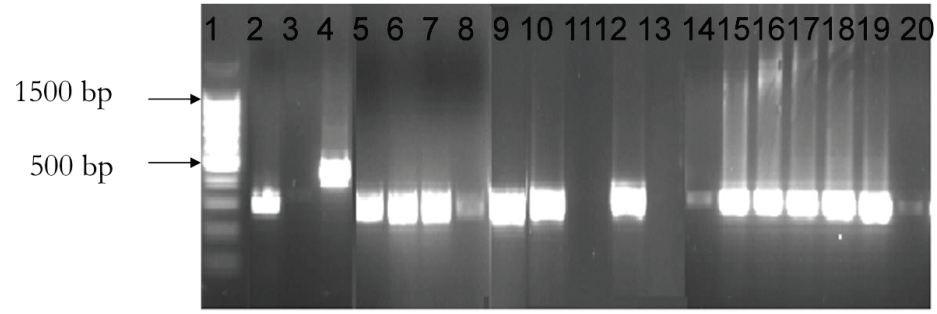

Figure 17. Electrophoretic separation of PCR products by RD4 deletion typing of mycobacteria isolated from smear positive sputum humans pulmonary tuberculosis patients. Lane 1, 100bp DNA ladder; Lane 2, H37Rv, positive control for *M. tuberculosis* ; Lane 3, water (negative control). Lane 4, 2122/97, positive control for *M. bovis,* Lanes 5-10,12 and14-20 were positive for *M. tuberculosis*., Lane 11 and 13 were negative samples

The mean age ± standard deviation of the study participants was 34 ± 14.8years with range from 10years to 84years, and 43% of the isolates were obtained from female and 57% were obtained from male patients. Majority (87%) of the study participants were Afar in their ethnicity living as pastoralists. The isolates were obtained from 11 different districts of the region, of which the predominant isolates were from Dubti District (50%), Amibara District (28%) and Awash District (14%) (Figure 1 in Manuscript IV).

4.4.2. Identification of strains of *M. tuberculosis* and *M. bovis* using spoligotyping

A total of 59 different spoligotype patterns (strains) were identified from the 150 *M. tuberculosis* complex isolates. Out of these 59 patterns, 27 (45.8%) were new and did not match with the existing patterns in the international SpolDB4 database. The new spoligotype patterns were designated as New1-27 (Table 1 in Manuscript IV). The two *M. bovis* were identified to be SB1519 based on www.mbovis.org database and were isolated for sputum of pulmonary TB patients in Awash District. Both *Mycobacterium bovis* isolated from pulmonary TB patients had identical spoligotype pattern and were epidemiologically linked in which the patient live in the same village of Awash District.

Based on the spoligotyping patterns, 74.7% (112/150) of the isolates were clustered into 9 spoligotype clusters each consisting 2 to 19 isolates in the clusters while 25.3% (38/150) were non-clustered spoligopattern having only single isolate. Majority (66.7%) of the strains did belong to Euro-American lineage followed by Indo-oceanic lineage (20.7%). Interestingly, 11.3% (17/150) isolates did not belong to any of the known lineage and classified as unknown lineage in the database (Figure 2 in Manuscript IV). Thus, 67.6% (100/148) of the isolates were in modern lineage and 32.4% (48/148) of them were ancestral lineage. All the isolates in unknown lineage were ancestral lineages (Table 1 and Figure 2 in Manuscript IV). The most frequent spoligotype patterns were SIT52, SIT149, new isolate (named as New3) consisting of 12.7% (19/150), 8.7% (13/150) and 8% (12/150) isolates, respectively. The new isolate which have been designed as New3 had very close spoligotype pattern with SIT149, the most common strain in Ethiopia, except that the spacer 17 was present in the New 3 while it is absent in SIT149.

From community-based active case detection study, four of the pulmonary patients had isolates with SIT1088 and epidemiologically the patients were close neighbors living in the same village. The patients also belong to the same Afar clan, which implies very close families and had intimate social interaction in the village exposing them for possibility of circulations of the strain in the families.

4.4.3. Identification of the strains of *M. tuberculosis* and *M. bovis* using MIRU-VNTR 24-loci typing

The MIRU-VNTR 24-loci typing were carried out on selected 48 isolates mainly based on their clustering in spoligotyping analysis. Out of these, two isolates were excluded from the final analysis because of the absence of PCR amplification and presence of multiple double alleles in more than two loci during MIRU-VNTR typing. Hence, the final analysis of MIRU-VNTR 24-loci typing was done on 46 isolates and identified 26 distinct MLVA MtbC15-9 types of which 13 of the patterns were clustered and consisted of 70% (32/46) of the isolates. The rest 13 patterns were not clustered and consisted of 28% (13/46) of the isolates (Figure 3). Of the total 26 MLVA MtbC15-9 types characterized by MIRU-VNTR 24-loci analysis, 16 patterns were not found in the MIRU-VNTR*plus* database and were reported as new strains. These new patterns were submitted to the database and obtained new MLVA MtbC15-9 number according to the MIRU-VNTR*plus* nomenclature (Table 2 in Manuscript IV). Based on the MIRU-VNTR*plus* database, MLVA MtbC15-9 type 594-15 was the predominant clustered pattern with 10.42%, followed by MLVA MtbC15-9 type 12960-15 with 8.33% and both of the predominant clustered isolates had SIT149 spoligotype pattern. The SIT149 spoligotype pattern were discriminated into four MLVA MtbC15-9 types in which case all patterns were clustered. These clustered isolates were mostly circulating in the three districts of the Region (Awash, Dubti and Amibara districts). MLVA MtbC15-9 type 6576-15 and MLVA MtbC15-9 type 913-15 were also the next frequent clustered patterns with each having 6.25% proportions. These two MLVA MtbC15-9 types had SIT52 spoligotype pattern.

The *M. bovis* strains (SB1519) which were characterized by spoligotype method were further analysed with MIRU-VNTR 24-loci and one of the isolate showed MLVA MtbC15-9 type 12967-1055 pattern, which was a new strain reported by this study and has obtained the nomenclature from MIRU-VNTR*plus* database. The other strain of *M. bovis* showed a double allele at Locus 2996(MIRU-26) and Locus 2163b (QUB-11b) hence was not included in the final analysis.

4.4.4. Transmission of *Mycobacteria* species between pastoralist and their livestock (zoonotic and reverse zoonotic transmission)

In this study, two *M. bovis* were isolated and characterized using spoligotype and further confirmed by MIRU-VNTR 24-loci molecular techniques. Both of the isolates were isolated from pulmonary tuberculosis patients living in the same geographic location of Awash district. In Afar pastoral region, pastoralists have very close physical association with their livestock during milking, herding, at watering point and in the night. Moreover, pastoralists consume raw/unpasteurized animal products including milk and milk products, meat. The existence of such potential risk factors could result a transmission of *M. bovis or Mycobacterium tuberculosis* or other *Mycobacterium* species from animals to human or vise versa. The isolation and confirmation of *M. bovis* strain SB1519, which was previously isolated for cattle of Ethiopia (Biffa *et al.,* 2010) in human pulmonary tuberculosis patients clearly indicated the transmission of the isolate from animal to human through aerosol route of transmission.

On other hand, isolation and characterization of *M. tuberculosis* strain SIT149 from strong tuberculin reactor goat in Amibara district indicate the existence of reverse zoonosis in which *M. tuberculosis* could have transmitted from human tuberculosis patient to the goat. In the district where this isolate collected similar strain were characterized from human pulmonary TB patients. In whole Afar, specifically small ruminants have very close association with human; particularly young animals share the same shelter with their owners. Since the age of the goat was around 2years, there was high chance that the goat contracted the disease from human patient who have been infected in the neighborhood. In molecular characterization, of the isolates from human, high rate of clustering (70%) has been observed in PTB patients isolates. This was an indication of circulation of specific strain of *M. tuberculosis* within Afar pastoralist communities and clear indication of defect in the tuberculosis control program.

On the other hand, a high rate of non-tuberculosis mycobacteria were isolated and characterized in human (12.8%) (Manuscript IV) and in animals (Paper I, Paper II) using molecular techniques of multiplex PCR, GenoTyping HAIN method (Figure 18) and 16s rDNA sequencing method. The result showed the importance of these species in epidemiology of pulmonary tuberculosis patients and in animal tuberculosis in camel, goat and cattle.

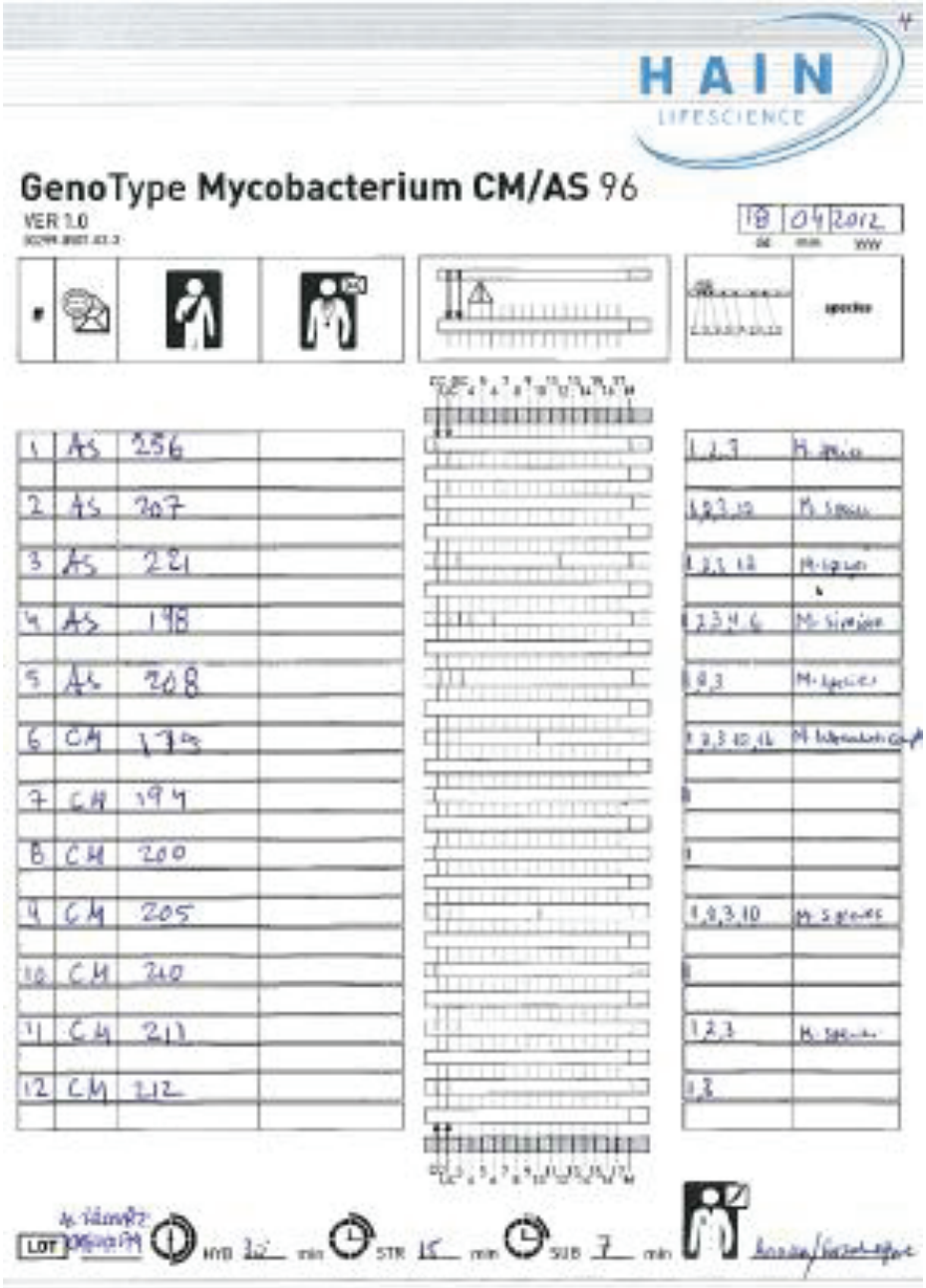

Figure 18. Representative result of isolates from human and animal characterized using GenoTyping HAIN method,

5. DISCUSSION

Tuberculosis remains a major global health problem causing high morbidity and mortality among millions of people each year. Two decades after the WHO declaration of TB as a global public health emergency in 1993, major progress has been made towards 2015 global targets of Millennium Development Goals (MDGs) in reduction of the burden of tuberculosis (WHO, 2013). However, in 22 high burden countries (HBCs) which accounted for 81% of all estimated incident cases worldwide, tuberculosis is still causing a serious public health problem. Ethiopia is one of the 22 HBCs with an estimated prevalence of all forms of TB 224 per 100 000 population and incidence rates of 247 per 100 000 population (WHO, 2013). On the other hand, Ethiopia being one of the countries with highest number of livestock resources in Africa, animal tuberculosis is known to be endemic and widespread in the country. However, in spite of high prevalence of both human and animal tuberculosis in the country, the emphasis given on tuberculosis to pastoral regions of the country, which constitutes 12-15 million of human population and possesses 42% of the country's livestock is very little.

Pastoralist communities whose livelihood relies on livestock and exposed to different risk factor are marginalized communities and information on molecular epidemiology of tuberculosis in the pastoralists and their livestock and the transmission pattern of *Mycobacteria* species among tuberculosis patients and different species of livestock and extent of zoonotic transmission of animal tuberculosis to human population or vice versa has not been well investigated.

In this thesis, an attempt was made to elucidate the epidemiology and pathology of tuberculosis in camels of pastoral regions of Ethiopia and molecular characterizations of the causative agents of camel tuberculosis (Paper I), investigation of the prevalence of tuberculosis and associated risk factors in small ruminants and cattle of Afar Pastoral Region and genetic diversity of the causative agents of livestock tuberculosis in the region (Paper II and III) and investigation of the molecular epidemiology of the etiology of tuberculosis in pulmonary TB patients of pastoralist community of Afar Region and genetic diversity of the of the causative agents of tuberculosis in human and role of *M. bovis* in human tuberculosis or *M. tuberculosis* in livestock were investigated in Afar region (Manuscript Paper IV).

The camel TB results that were based on postmortem examinations of 906 *Camelus dromedarius* was one of the largest camel TB studies of its kind in the world in terms of its sample size and identification of its etiology. Based on the results of the present study a moderately high prevalence (10%) of camel tuberculosis was obtained on camels of Ethiopia originated from south and northeastern pastoral regions of Ethiopia indicating a widespread distributions of the diseases in camels (Paper I). As the same time the prevalence was comparable with the result of recent study in Eastern Ethiopia (12.3%) (Zerom *et al.* 2013). On the other hand, the reported prevalence in the present study was higher than the previous abattoir based prevalence reports from Dire Dawa abattoir, Ethiopia (Mamo *et al.*, 2009) and Egypt (Refai, 1992). The high prevalence of camel TB in the present study might be related to the difference in geographic area of the study sites in which different risk factors of infection including close contact with other livestock might be different in the study sites. Similarly, based on comparative intradermal tuberculin test results on camels from Afar Region also showed a moderately high prevalence (16.6%) of bovine tuberculin reactors and camels in Amibara district showed the highest proportion of bovine tuberculin reactor than Chifra district, which might be associated with the fact that higher interactions of camel with other species of livestock (cattle and small ruminants) at grazing, watering and at night were observed in Amibara District than Chifra District. Moreover, most of livestock migrate from other districts to Amibara District at banks of Awash River during their seasonal migration.

In the present study among the host factors considered age was associated with the occurrence of TB lesions in camels in which lesions were predominantly found in the younger and older camel. Similarly, other studies also reported in cattle particularly that older animals are affected by TB (Kazwala *et al*., 2001; Cleaveland *et al.*, 2005; Inangolet *et al*., 2008), which could be due to longer duration of exposure to the causative agent. Similarly, comparative intradermal tuberculin test result on Afar camels also revealed that older camels had the highest prevalence as compared to the younger camels, which supports result of the postmortem examination.

The higher frequency of lesion in younger camels could due to the less developed immunity. Young camels can also be easily infected with higher doses of mycobacteria via colostrums from infected camel in a similar way, as it occurs in cattle (Phillips *et al*., 2003). In connection to this, another report mentioned of vertical transmission of *M. bovis* from an infected dam to her calf through congenital infection in uterus (Ozyigit *et al*., 2007). In the present study, it was also observed that tuberculous lesions were more frequently observed in

female camels as compared male camels. This could be because female camels were brought for slaughter at their oldest ages after completion of the reproductive age, which therefore can be attributed to the longer duration of exposure to the causative agent (Paper I).

Regarding the pathology of tuberculous lesions in camel, the mesenteric lymph lodes were found to be the most frequent and severely affected of all the lymph nodes, indicating the oral route was the main route of infection in camels of pastoral regions of Ethiopia and the result was similar with reports from cattle under extensive livestock production system (Ameni *et al.* 2007; Ameni *et al.,* 2010).

The main finding of the present camel tuberculosis study was the isolation and molecular characterization of the causative agents camel tuberculosis in Ethiopia (Paper I). According to the results, of the two *M. bovis* isolated and characterized using RD-4 deletion typing and spoligotype, one of the *M. bovis* (SB1953) was a new strain of *M. bovis* reported for the first time to www.Mbovis.org database by this study. The identification of this new *M. bovis* strain from camel TB case of pastoral area of Ethiopia imply the existences of large diversity of spoligotype patterns in terms of host and geographical area of the country. On the other hand, the isolation of SB0133 *M. bovis* strain in present study from camel of pastoral area of Ethiopia, in line with the isolation of this strain from cattle of southern Ethiopia (Berg *et al.*, 2009; Biffa *et* al., 2010) and pastoral area of Uganda (Oloya *et al.,* 2006) indicates the predominant localization of the strain to pastoral regions of Eastern Africa and possible interspecies transmission of the strain among livestock of pastoral area. In addition, the development of TB lesions to generalized disseminated form of TB in camel affected by SB0133 strain might imply its high pathogenicity in camels.

The other main finding in camel tuberculosis study was isolation of 18 non-tuberculous mycobacteria species from typical tuberculous lesions and further characterization using 16SrDNA sequencing showed that the majority (77%) of the NTM isolated from camel lesions were members of *M. terrae* complex and the rest were *M. flavescens*, *M. acapulcensis*, *M. chelonae*, *M. moriokaense* and *M. avium*. This is the first report of characterization of these species of mycobacteria from camel in Ethiopia. Similar previous studies on cattle and goat in extensive production system and wild animals in Ethiopia have also characterized different species of NTM as the causative agents (Ameni *et al.*, 2008; Berg *et al.*, 2009; Tschopp *et al.*, 2010; Gumi *et al.,* 2012). The fact that these species were predominantly isolated from camels, cattle, goat and wild animals in Ethiopia suggests the

importance of NTM in epidemiology of animal tuberculosis in Ethiopia and the need for further investigation on their pathogencity and transmission pattern in these species. In addition, the relatively higher portion of avian tuberculin reactor in camels obtained on comparative intradermal tuberculin test result of this study might be also related to the existence and extent of infection by NTM in camel of pastoral regions of Ethiopia.

Tuberculosis in goat and sheep is not well studied in Ethiopia particularly in pastoral regions of the country, which harbors 70% of goat and sheep population of the country (PFE, 2010b; Shitarek, 2012). In this thesis (Paper II), an extensive tuberculosis study on small ruminants of Afar Pastoral Region based on comparative intradermal tuberculin test showed that an overall low prevalence of tuberculosis (0.5% at 4mm cut-off point and 3.8% at 2mm cut-off point) in goat and sheep of the region. The result is in agreement with other studies in Ethiopia which were carried out based on abattoir examination and skin test results and the prevalence in this studies ranged from 0.2% to 4.2% (Tschopp *et al.,* 2011; Hiko and Agga, 2011; Tafess *et al.*, 2011; Gumi *et al.,* 2012; Deresa *et al.*, 2013). Hence, the result of this study and others showed that the overall prevalence of tuberculosis in goat and sheep in general is low in Ethiopia. The proportion of positive reactors was significantly higher in Dubti district than the other districts, which might be related to the husbandry system where small ruminants had higher interaction with cattle in Dubti districts than the other districts, which can favor a potential transmission of mycobacterial species between cattle and goat. Older goat and sheep showed higher proportion of positivity in tuberculin test results which might be related to the fact that older animals have longer duration and repeated chance of exposure to mycobacterial infection with their age. Similar results have been reported by other researchers in cattle (Ameni *et al.*, 2007; Cadmus *et al.* 2010).

The main finding of this study was the isolation and characterization of *M. tuberculosis* and NTM from tuberculin reactor goats. In this study, *Mycobacterium tuberculosis* strain SIT149 was isolated from a goat suggesting the possibility of its transmission from human to goat. The SIT149 strain of *M. tuberculosis* is a dominant strain in Ethiopia (Brudey *et al.,* 2006) and it was a common isolate in human pulmonary TB patients from the same Afar Pastoral Region indicating that the isolate has been circulating in the area. Afar pastoralists have close contact with goats and sheep and often keep young goats and sheep in their house at night,

which might be a potential factor for transmission from human patient to animals (reverse zoonosis). Similar strain has been isolated in camel from pastoral region in south east of Ethiopia (Gumi *et al.,* 2012). Previous studies in cattle of Ethiopia also demonstrated that *M. tuberculosis* was commonly isolated from tuberculous lesions of cattle in different regions of Ethiopia (Berg *et al.,* 2009; Ameni *et al.*, 2010).

In Ethiopia, in spite of large population of cattle and endemic nature bovine tuberculosis, very few studies were carried out to investigate the epidemiology and associated risk factors with the diseases in pastoral regions of the country. In the present study which is the first of its kind in Afar Region , a moderately high prevalence (11% at 4mm of cut-off point and 18% at 2mm of cut-off point) of BTB in cattle was reported (Paper III) which indicate the endemic nature of BTB in cattle of the Region. The overall prevalence was higher than previous reports from other pastoral areas of Ethiopia (Tschopp *et al.,* 2010; Gumi *et al.*, 2011) and Uganda (Inangolet *et al.*, 2008). The difference might be related to the epidemiological factors that favors the transmission of BTB in the Afar Region, which include large herd sizes, communal grazing and watering of diverse species of animals including camel, cattle, goat and sheep, and an extensive seasonal mobility within and outside the districts, which creates favorable condition for close contact with other livestock and wild animals such as Oryx, antelope and warthog. The possibility of transmission of *M. bovis* between wildlife and cattle has been reported from other part of Africa and Europe (Woodford, 1982; Phillips *et al*., 2003; Cleaveland *et al*., 2005).

Moreover, every year during the months of November to February, large number of livestock from different districts of Afar Region congregate in cotton irrigation farms after the cotton is harvested to graze on the leftover of cotton farm create a favorable condition for close contact between animals and potential risk for transmission of diseases such as BTB among the animals. Such epidemiologically conducive conditions could lead to higher prevalence of BTB in the Afar Region as compared to the prevalence in other pastoral regions in Ethiopia.

On the other hand, the result of the present study was much lower than the higher prevalence of BTB reported in urban intensive dairy farms of Ethiopia (Ameni *et al*., 2003, Ameni *et al.*, 2007). This difference might be mainly related to the intensive husbandry system practiced and the breed susceptibility (Ameni *et al*., 2007; Tsegaye *et al*., 2010). In our study, the

animals tested were zebu Afar breed of cattle managed under extensive pastoral husbandry system which might be a possible reason for the differences in result, as the zebu breeds are known to be relatively resistant to BTB as compared to Holstein and other cross breeds managed under intensive system (Ameni *et al*., 2007, Cadmus *et al*., 2010).

The prevalence of BTB showed an increase with age and this finding was in agreement with previous reports by others (Kazwala *et al*., 2001; Ameni *et al*., 2007; Inangolet *et al*., 2008; Regassa *et al*., 2010, Cadmus *et al.*, 2010; Biffa *et al*., 2011). As indicated by these authors, the possible reasons could be the fact that older animals had longer and repeated chance of exposure to mycobacterial infection during their life time.

Furthermore, it has been observed that cows were more positive reactor than bulls, which is in agreement with other studies (Inangolet *et al*., 2008; Cadmus *et al*., 2010). In the Afar pastoral system, the majority (90%) of their herds is composed of cows kept exclusively for milk production and kept for longer time than the bulls, which form less than 10% of the herd, as bulls are sold or slaughtered in their early age. This condition might be the reason for higher tuberculin positivity in cows than bulls.

In the molecular epidemiology study of Mycobacteria isolates obtained from human pulmonary TB patients of Afar Region, a total of 148 *M. tuberculosis* and two *M. bovis* isolates were recovered and identified. Majority of the *M. tuberculosis* isolates were found in clusters both up on analysis using spoligotyping (74.7%) and MIRU-VNTR typing (70%) methods which was in agreement with other studies in Ethiopia (Diriba *et al.*, 2013), in North Malawi (Glynn *et al.*, 2005), in South Africa (Verver *et al.,* 2004) and in Taiwan (Dou *et al.,* 2008) with frequencies of 83%, 72%, 72%, and 67%, respectively. Such high rate of clustering suggests a circulation of specific strains in a population and existence of recent transmission in the study population. On the other hand, the result of clustering was inconsistence with other reports in different regions of Ethiopia which have reported lower clustering frequencies, 45% and 46% in Northwest Ethiopia (Tessema *et al.,* 2013; Debebe *et al.,* 2013) and 42.1% in Addis Ababa city (Bruchfeld *et al.,* 2002). The difference might be related to the difference in social organization and cultural practices of the study population in which the Afar pastoralist communities which have unique social and cultural homogeneity that creates a high rate of close contact with active cases combined with high

pulmonary TB in Afar Region (WHO, 2009) favoring transmission of similar strains resulting higher rate of clustering.

In this study a high genetic diversify were observed with 59/150 different spoligotype patterns and 26/46 different MLVA MtbC15-9 types. The result was in agreement with a previous study on pulmonary TB patients in neighboring Djibouti, a country that has a similar social and demographic profile with that of Afar Region of Ethiopia (Godreuil *et al.* 2009). In addition, out of the 59 spoligotype patterns, 27 patterns were new to SITVIT spoligotype database, and similarly out of the 26 MLVA MtbC15-9 types obtained in MIRU-VNTR 24-loci analysis 16 patterns were new to MIRU-VNTR*plus* database. This could suggests that the circulation of wide genetically variable strains of *M. tuberculosis* in the Region. The existence of these highly diversified strains of *M. tuberculosis* could imply the existence of a process of evolutionary cloning in long period. This could be a result of failure in TB control program, which has allowed free circulation of strains, and their evolvement in the population thereby leading to the formation of such genetic diversity (Godreuil *et al.* 2009).

The most common spoligotype patterns in the study were SIT52, SIT149, new strain (named in this study as New3). The former two strains were reported in previous studies (Tessema *et al.*, 2013; Firdessa *et al.,* 2013). Similarly, further typing of SIT149 using MIRU-VNTR 24 loci resulted in identification of four different patterns of MLVA MtbC 15-9 type of which MLVA MtbC15-9 type 594-5 was the predominant strain followed by MLVA MtbC15-9 type 12960-15. This clearly shows ongoing recent transmission of TB by SIT149 strain in the study area. Similar finding was reported from northwest Ethiopia where MLVA MtbC15-9 type 594-5 was identified to be the predominant strain causing TB in the area (Tessema *et al.,* 2013). The new strain which have been designated as New3 had very similar spoligotype pattern with SIT149, except that the spacer 17 was present in the New 3 while it is absent in SIT149. This could suggest the possibility that SIT149 might have evolved from the New-3 strain.

Interestingly in this study, the two *M. bovis* isolates were characterized using spoligotype and further confirmed by MIRU-VNTR 24-loci analysis. This is an important finding that showed the public health importance of *M. bovis* in pastoralist communities of the Afar Region, northeast Ethiopia. Similar finding was reported from Borona pastoral communities in the southern Ethiopia (Gumi *et al.,* 2012). Different from previous study (Firdessa *et al.,* 2013) which reported minimal role of *M. bovis* as a cause of TB in humans in Ethiopia, these two

studies in pastoral areas could suggest potential risk of *M. bovis* to cause TB in pastoralists. In addition, the fact that the *M. bovis* isolates were detected from active smear positive pulmonary TB patients from the same geographic localities is of great concern as the possibility of aerosol transmission from human to human might be one potential route of transmission in the region once it escapes from livestock host and need further investigation.

Euro-American lineage was the predominant lineage (67%) followed by the most ancestral Indo-Oceanic lineage (21%) consisting of mainly CAS, EAI sub-lineages. This result was in agreement with previous studies carried out in different regions of Ethiopia (Debebe *et al.,* 2013; Firdessa *et al.,* 2013; Mihret *et al.,* 2012). On the other hand, 11.3% of the isolates could not be classified into the known lineages in the database although they belong to the ancestral lineage group according to the SPOTCLUST database. The finding of these ancestral lineage combined with the fact that the Afar Region was the origin of early hominids (Johanson and White, 1979; Asfaw *et al,* 1999; White *et al,* 2009) might support the suggestion on co-evolution of ancestral mycobacteria with their human host in the horn of Africa (Hirsh *et al.,* 2004; Gutierrez *et al.*, 2005; Gagneux *et al.,* 2006; Wirth *et al.,* 2008). In addition, ancestral species of *Mycobacterium* such as *M. canetti* (van Soolingen *et al.,* 1997; Pfyffer *et al.*, 1998) and the most ancestral lineage of the six known lineage, Indo-oceanic lineage were associated with East Africa origin (Gagneux *et al.,* 2006).

5.1. Limitations of the thesis

This thesis provides data important information on molecular epidemiology of tuberculosis in pulmonary TB patients of Afar pastoralist communities and their livestock and transmission pattern among different species livestock and human has been addressed. Moreover, in this thesis, *Mycobacteria* species isolated from camel, goat, cattle and human patients from pastoral region were molecularly characterized to establish the genetic diversity of the *Mycobacteria* species in Afar Pastoral Region and understand the pattern of transmission between human and other domestic livestock. However, the thesis has limitation related to study site selection, sampling methods and obtaining of laboratory tissue samples from cattle.

5.1.1. Selection of the study sites and study animals

For epidemiological study of tuberculosis in livestock, the study districts and subdistricts should have been selected using the simple random method, which would increase the full representativeness of the selected districts and subdistricts. However, because of inaccessibility, insecurity and lack of laboratory facilities to collect and store collected

samples security, the study was conducted only in four selected districts out of the 32 districts in Afar Regional State, which were selected based on convenient method.

On other hand, selection of animals for comparative intradermal tuberculin test, since there is no animal recording system, selection of the animals involved convenient method for the owner. Therefore, this could be considered as one of the limitation of the study related to the epidemiology of tuberculosis in livestock in the area.

5.1.2. Tissue sample collection for mycobacteria isolation from cattle

In the Afar Region, there is no abattoir and hence collection tissue samples from cattle were not possible. The possibility of collecting tissue samples from tuberculin reactor cattle by purchasing and slaughtering these BTB positive animals was not possible because of economic constraints. Alternatively, attempt to isolate *Mycobacteria* species from milk and nasal swab of tuberculin reactor animals was not conclusive since mycobacteria might not shed through milk or nasal discharge in tuberculin reactor animals unless it involved extensive disseminated lesions in the udder and lungs. On the other hand, isolation of NTM form milk and nasal swab of tuberculin reactor animals might also involve contamination from the environment during sample collection.

5.1.3. Laboratory related limitations

In the molecular characterization of *Mycobacteria* isolates from pulmonary tuberculosis patients, out of the 172 isolates, which were characterized, by spoligotyping only 48 isolates were analyzed further using MIRU-VNTR analysis method because of shortage of MIRU-VNTR genotyping kit. Hence, this can be taken as on limitations of the study in the interpretation of the genotype and clustering rate of the whole isolates from human patients. Similarly, the NTM characterized at genus *Mycobacterium* level using multiplex PCR and HAIN Genotyping methods from human PTB patients and animals should have been further characterized to species level using 16S rDNA sequencing method to identify the specific species of *Mycobacteria* causing the disease. Finally, the *Mycobacteria* isolates from human patients were only from pulmonary patients and extrapulmonary TB cases were not included in this study. This is due to lack of specialized pathologist to collect extrapulmonary samples (fine-needle aspirate, pleural effusion or biopsy sample from tuberculous lymphadenitis cases), hence this can be considered as one limitation of the study.

Table 5. Major findings, Implication and future research plan based on the result of this study

Major findings	**Implications**	**Future research plans**	**Evidences**
• Bovine tuberculosis is widespread in Afar Pastoral Region with moderately high prevalence in cattle and camel and with low prevalence in small ruminants	Endemic nature of BTB in pastoralist of Ethiopia	Research on identification of the reservoir of BTB and their role, transmission pattern, design of feasible control options	**Paper I, II, III**
• *M. bovis* -SB1593 (New strain reported by this study), *M. bovis* -SB0133, *M. terrae* complex, *M. flavescens*, *M. acapulcensis*, *M. chelonae*, *M. moriokaense* and *M. avium*. were the causative agents of tuberculosis in camel	*M. bovis* as cause of camel tuberculosis and NTM are the major causes of tuberculosis like diseases in camel	Zoonotic significance of the causative agents should be investigated and transmission pattern should be researched	**Paper I**
• *M. tuberculosis* strain SIT149 has been identified as a causative agent of tuberculosis in a goat, which implies the potential transmission of the strain from human patient.	Reverse zoonosis and small ruminants can act as reservoir of M. *tuberculosis* for human infection, Zoonotic transmission is a possibility	Transmission dynamics should be well investigated and control of TB in small ruminants should be considered for control of TB in human population	**Paper III**
• High genetic diversity of *M. tuberculosis* in PTB of the pastoral community • High rate of clustering of strains observed • Two *M. bovis* isolates were isolated from PTB patients and zoonotic transmission observed • Sixteen new strains of Mtbc 15-9 MIRU-VNTR have been reported to the MIRU-VNTRplus database. • Twenty seven new spoligotype patterns were reported from this study • NTM were found to cause clinical tuberculosis in pastoralist community	Tuberculosis control program has defect as recent transmission and circulation of strains is observed , reexamining the strategy of TB control program is critical in pastoralist area; Zoonotic transmission is evident and aerosol route of transmission is the significant pathway, Education of the pastoralist on the risk of zoonotic transmission; Potential human to human transmission through aerosol route is evident; Drug efficacy must be examined	Case tracing study using molecular method should be carried out, transmission route should be identified, Extrapulmonary TB cases should be investigated, Sequencing of the NTM isolates and their transmission dynamics should be investigated	**Manuscript IV**

6. CONCLUSION

In the this dissertation, we found that there was high genetic diversity of *Mycobacterium tuberculosis* in human pulmonary tuberculosis patients with high rate clustering of the strains of *M. tuberculosis* indicating the circulation of specific strains in Afar pastoralist community which indicates the defect of the TB control program in the region. The isolation and molecular characterization of *M. bovis* from sputum sample of human pulmonary tuberculosis patients shows the zoonotic significance of *M. bovis* in the pastoralist community of the study area. In the present study, we found also the endemic nature of tuberculosis in camel, cattle and small ruminants of pastoralists' community of Afar. The bovine tuberculosis is wide spread affecting different species of animals (camel, cattle, goat and sheep) a moderately high prevalence of tuberculosis in camel and cattle of pastoral regions. On the other hand, identification of *M. tuberculosis* strain SIT149 from tuberculin reactor goat from pastoralist area showed that the potential reverse transmission and infection of livestock by *M. tuberculosis* originated from human. This has an important implication in the control program of tuberculosis in human as livestock may act as reservoir of the *M. tuberculosis* in pastoralist setting of Ethiopia. The present study also revealed the importance of non-tuberculosis mycobacteria as a cause of tuberculosis-like diseases in animals and humans of Afar pastoral region. A number of species of non-tuberculosis mycobacteria were isolated and characterized from camel, goat and cattle. However, the extent their pathogencity, transmission pattern and drug susceptibility need to be further investigated.

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Pathology of Camel Tuberculosis and Molecular Characterization of Its Causative Agents in Pastoral Regions of Ethiopia

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Abstract

A cross sectional study was conducted on 906 apparently healthy camels slaughtered at Akaki and Metehara abattoirs to investigate the pathology of camel tuberculosis (TB) and characterize its causative agents using postmortem examination, mycobacteriological culturing, and multiplex polymerase chain reaction (PCR), region of difference-4 (RD4)-based PCR and spoligotyping. The prevalence of camel TB was 10.04% (91/906) on the basis of pathology and it was significantly higher in females ($\chi^2 = 4.789$; $P = 0.029$). The tropism of TB lesions was significantly different among the lymph nodes ($\chi^2 = 22.697$; $P = 0.002$) and lung lobes ($\chi^2 = 17.901$; $P = 0.006$). Mycobacterial growth was observed in 34% (31/91) of camels with grossly suspicious TB lesions. Upon further molecular characterization using multiplex PCR, 68% (21/31) of the colonies showed a positive signal for the genus *Mycobacterium*, of which two were confirmed *Mycobacterium bovis (M. bovis)* by RD4 deletion typing. Further characterization of the two *M. bovis* at strains level revealed that one of the strains was SB0133 while the other strain was new and had not been reported to the *M. bovis* database prior to this study. Hence, it has now been reported to the database, and designated as SB1953. In conclusion, the results of the present study have shown that the majority of camel TB lesions are caused by mycobacteria other than *Mycobacterium tuberculosis* complex. And hence further identification and characterization of these species would be useful towards the efforts made to control TB in camels.

Citation: Mamo G, Bayleyegn G, Sisay Tessema T, Legesse M, Medhin G, et al. (2011) Pathology of Camel Tuberculosis and Molecular Characterization of Its Causative Agents in Pastoral Regions of Ethiopia. PLoS ONE 6(1): e15862. doi:10.1371/journal.pone.0015862

Editor: Niyaz Ahmed, University of Hyderabad, India

Received August 24, 2010; **Accepted** November 25, 2010; **Published** January 24, 2011

Funding: This study was financially supported by the Norwegian Programme for Development, Research and Education (NUFU), PRO-2007/10198. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Pastoral production system accounts for the livelihood of 50–100 million people in developing countries and 60% of this population lives in more than 21 African countries confined to the most arid regions of the continent [1,2]. In eastern Africa, Ethiopia has the largest pastoralist population (7–8 millions) representing around 20 ethnic groups [3]. The major ethnic groups in Ethiopia are Somalis, Afar, Kereyu and Borena pastoral communities occupying the Eastern and southern lowlands of the country. Pastoralist depends on livestock for their livelihood, moving seasonally from place to place in search of water and pasture for their animals [4]. The dromedary camel (*Camelus dromedarius*), which is a versatile animal capable of living in harshly semi-arid and arid areas of the world, is extremely important for livelihood of pastoral communities through provision of milk, meat and draft power for transportation of goods. In pastoral communities of Afar, Somali and Borena, camels are kept almost entirely for milk production [5]. In these communities, camel milk is consumed raw, and this habit combined with close physical contact with their animals create a potential public health concern for transmission of zoonotic diseases such as tuberculosis (TB) from animals to the pastoralist.

Although, the extent of TB has been well documented in humans and most domestic animals, very little is known about the pathology and cause of camel TB in pastoral areas of the world. Camel TB has been reported in Egypt [6], United Arab Emirates [7,8], Pakistan [9], and Australia [10]. *Mycobacterium tuberculosis (M. tuberculosis)*, *Mycobacterium bovis* (*M. bovis*), and atypical mycobacteria such as *Mycobacterium kansasii* (*M. kansasii*), *Mycobacterium aquae* (*M. aquae*), *Mycobacterium fortuitum* (*M. fortuitum*) and *Mycobacterium smegmatis* (*M. smegmatis*) have been isolated in camel as causative agents of camel TB [8,11]. In Ethiopia, except one report indicating the existence of camel TB [12], there is a large paucity of information on the pathology and the causative agent of TB in camels of pastoral regions of the country. Therefore, investigation of the pathology of camel TB and identification of its causative agents is important to encourage the effort in the control of the disease and reduce its risk of zoonosis to the pastoralist community of Ethiopia. The present study, therefore, was designed to investigate the pathology of camel TB and identify the causative agent using molecular tools.

Materials and Methods

Study Animals

The cross sectional study was carried out on 906 apparently healthy male (n = 535) and female (n = 371) slaughtered camels.

The camels slaughtered were brought to Akaki (Addis Ababa) and Metehara Abattoirs from the two main pastoral regions of Ethiopia, namely Awash-Fentale pastoral area (Kereyu and Afar in the Middle Awash region) and Borena pastoral area (southern Ethiopia). The catchment areas possess large number of camels, in Fentale pastoral area (Middle Awash region) there are 68,331 camels and in Borena pastoral area of Oromia Regional State which border with Kenya possesses an estimated population of 97,131 camels [13]. After arriving at the abattoir, the camels were staying for 2–7 days undergoing physical examination. On average 6–8 camels were slaughtered per day depending on the request from customers. The main consumers of camel meat in Addis Ababa are the Somali immigrants residing in the city.

Post mortem inspection and pathology scoring

Postmortem inspection was performed following the procedure as previously described [14]. Mandibular, retropharyngeal, bronchial, mediastinal, mesenteric and hepatic lymph nodes were examined and organs including lungs, liver, small intestine and kidneys were examined in detail during post-mortem in the abattoir under a bright-light source. The lobes of the left and right lungs were inspected and palpated externally. Then, each lobe was sectioned into about 2-cm-thick slices to facilitate the detection of lesions with sterile surgical blades. Similarly, lymph nodes were sliced into thin sections (about 2mm thick) and inspected for the presence of visible lesions. Whenever gross lesions suggestive of TB were detected in any of the tissue, the tissue was classified as having lesions.

Pathology scoring was conducted on tissues with abscesses and tubercle lesions to determine the severity of the lesions based on semi quantitative procedure developed previously [15,16]. Briefly, lesions in the lobes of the lungs were scored separately as follows: 0 = no visible lesions; 1 = no gross lesions but lesions apparent on slicing of the lobe; 2 = fewer than five gross lesions; 3 = more than five gross lesions; 4 = gross coalescing lesions. The scores for the individual lobes were summed and generated lung score. Similarly, the severity of gross lesions in individual lymph nodes was scored as follows: 0 = no gross lesions; 1 = small lesion at one focus; 2 = small lesions at more than one focus; 3 = extensive necrosis. Individual lymph node scores were summed and generated the lymph node score. Total pathology score per animal was obtained from the sum of the two total scores.

Mycobacterial isolation from tissue lesions

For mycobacteriological isolation tuberculous lesions from slaughtered camels were aseptically collected into sterile universal bottles with about 5 ml of 0.9% saline solution and also kept in icebox with solid packs to keep the cold chain. Then the samples were transported to Aklilu Lemma Institute of Pathobiology (ALIPB) and stored at +2 to +8°C until mycobacteriological culturing was carried out in TB laboratory.

The samples were further processed for isolation of mycobacteria in accordance with the Office International des Epizooties [17,18]. The specimens were sectioned using sterile blades, minced with scissors and homogenized with a sterile mortar and pestle under a biological safety cabinet. The homogenates were decontaminated by adding an equal volume of 4% NaOH on the sample in order to remove contaminants. Thereafter, centrifuged at 3,000 rpm for 15 minutes to concentrate the mycobacteria. The supernatant was discarded, and the sediment was neutralized by 1% (0.1 N) HCl acid using phenol red as an indicator. Neutralization was achieved when the color of the solution changed from purple to yellow [17]. Next, 0.1 ml of suspension from each sample was spread onto a slant of Löwenstein Jensen (LJ) medium. Duplicate slants were used, one enriched with sodium pyruvate and the other enriched with glycerol. Cultures were incubated aerobically at 37°C for 8–12 weeks with weekly observation for growth of colonies. Positive cultures were confirmed with Ziehl Nelseen staining and preserved with freezing media while at the same time heat killed in water bath at 80°C for 45 minutes. The frozen and heat killed isolates were stored at (−20°C) for further mycobacteriology and molecular typing analysis.

Mycobacterial genus typing

The multiplex PCR differentiate *M. tuberculosis* complex from *M. avium* complex, *M. intracellularae* and other mycobacterial species. Mycobacterial genus typing was conducted as described previously [19]. Heat killed AFB positive samples were used as source of DNA template.

DNA amplifications was done in thermocycler with 20 µl reaction volumes consisting: 5 µl of genomic DNA as a template, 8 µl HotstarTaqMasterMix (MgCL2, dNTP, Taq polymerase and PCR buffer) (Qiagen, United Kingdom) for each sample, 0.3 µl internal primer per sample, 0.3 µl forward and reverse primer per each sample and 5.2 µl per sample of Qiagen water. The primers used for amplification were MYCGEN-F, 5′AGA GTT TGA TCC TGG CTC AG 3′ (35ng/µl); MYCGEN-R, 5′TGC ACA CAG GCC ACA AGG GA 3′ (35ng/µl); MYCAV-R, 5′ ACC AGA AGA CAT GCG TCT TG 3′(35ng/µl); MYCINT-F, 5′CCT TTA GGC GCA TGA TGT CTT TA 3′(75ng/µl); TB1-F, 5′ GAA CAA TCC GGA GTT GAC AA 3′ (20ng/µl); TB-1-R, 5′ AGC ACG CTG TCA ATC ATG TA 3′ (20ng/µl). *M. tuberculosis* strains (H37Rv) and *M. avium* were used as positive control while Qiagen water was used as negative control. The reaction mixture was then heated in Programme Thermal Controller (Applied biosystem; PTC- 100^{TM}) cycle using the following amplification program: 95°C for 10 minutes for enzyme activation; 95°C for 1 minute for denaturation; 61°C for 0.5 minute for annealing; 72°C for 2 minutes for extension, involving 35 cycles all in all; and final extension at 72°C for 10 minutes.

The products were electrophoresed in 1% agarose gel in 10× TAE running buffer. Ethidium bromide at ratio of 1: 10, 100bp DNA ladder, and orange 6× loading dye were used in gel electrophoresis. All members of the genus *Mycobacterium* produce a band of 1030bp, *M. avium* or subspecies such as *M. avium* subspecies *paratuberculosis* produces a band of 180bp, *M. intracellularae* a band of 850bp while members of *M. tuberculosis* complex produce a band with 372bp.

RD4 deletions typing

PCR analysis on the basis of RD regions has been found to be an important differentiating tool between members of the *M. tuberculosis* complex. RD4 is 12.7 kb genetic segment that is deleted from *M. bovis* BCG strain, but present in *M. microti*, *M. africanum*, and *M. tuberculosis* [20].

The RD4 deletion typing was carried out on isolates that showed band for *M. tuberculosis* complex by multiplex PCR. For this deletion typing, the procedure described by Cadamus and coauthors was followed [21]. Each sample was tested in a separate PCR tube. Primers directed against the RD4 were used to generate a deletion profile that would allow species identification of the isolate. Primers that were used include RD4intF ACA CGC TGG CGA AGT ATA GC, RD4flankF CTC GTC GAA GGC CAC TAA AG and RD4flankR AAG GCG AAC AGA TTC AGC AT to check for the presence of RD4 locus. The HotStarTaq Master Mix system from Qiagen was used for

Table 1. Logistic regression analysis of tuberculous lesions with various host-related risk factors.

Characteristics	No. examined	No. of positive (%)	Crude Odds ratio (95% CI)	Adjusted Odds ratio (95% CI)
Sex				
Female	371	46 (12.4)	1	1
Male	535	45(8.4)	0.62 (0.40–0.95)	0.64 (0.36–1.2)
Age				
<4	99	12 (12.1)	1	1
4–6	184	14 (7.6)	0.60 (0.26–1.35)	0.25 (0.26–1.35)
7–9	141	10 (7.1)	0.55 (0.23–1.34)	0.54 (0.22–1.32)
10–15	197	19 (9.6)	0.77 (0.36–1.67)	0.64 (0.28–1.46)
16+	285	36 (12.6)	1.05 (0.52–2.11)	0.74 (0.32–1.68)
BCS				
Poor	389	44 (11.3)	1	1
Medium	330	36 (10.9)	0.96 (0.60–1.53)	0.92 (0.57–1.47)
Good	187	11 (5.9)	0.46 (0.25–.0.97)	0.42 (0.20–0.86)
Origin				
Kereyu	609	56 (9.2)	1	1
Borena	297	35 (11.8)	1.32 (0.84–2.06)	1.24 (0.70–2.2)

CI = Confidence Interval; BCS = Body Condition Scoring; odds ratio corresponding to different categories of a given variable are adjusted for the remaining three variables.

doi:10.1371/journal.pone.0015862.t001

PCR, with primers described previously. The reaction mixture was 10 µl of HotStarTaq Master Mix, 0.3 µl×3 of each primer (flank R, F and int), 2 µl DNA template and 7 µl distilled water to a final volume of 20 µl. *M. tuberculosis* H37Rv and *M. bovis* 2122/97 were used as positive control while Qiagen water was used as negative control. The mixture was heated in Programme Thermal Controller (Applied biosystem; PTC- 100^{TM}) using an initial hot start of 95°C for 15 minutes, followed by 35 cycles of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute; a final extension step of 72°C for 10 minutes to complete the cycle. PCR products were electrophoresed in 1% agarose gel in 1× TAE running buffer, Ethidium bromide at ratio of 1: 10, 100bp DNA ladder and orange 6× loading dye were used in electrophoresis. The gel was visualized in Multi–image^{TM} light cabinet using Alpha innotech version 1.2.0.1(Alpha Innotech Corporation). The presence of RD4 (*M. tuberculosis, M. africanum*) gives a product size of 335bp (RD4int+RD4FlankR) and its absence (*M. bovis*) gives a product size of 446bp (RD4FlankR+RD4FlankF).

Spoligotyping

Spoligotyping was performed as previously described by Kamerbeek and coauthors [22] and according to the spoligotype kit supplier's instructions (Ocimum Biosolutions Company, Iisselstein, The Netherlands). The direct repeat (DR) region was amplified by PCR using oligonucleotide primers derived from the DR sequence. A total volume of 25 µl the following reaction mixture was used for the PCR: 12.5 µl of HotStarTaq Master Mix (Qiagen: this solution provides a final concentration of 1.5 mM MgCl2 and 200µM of each deoxnucleotides triphosphates), 2 µl of each primer (20 pmol each), 5 µl suspension of heat-killed cells (approximately 10 to 50ng), and 3.5 µl distilled water. The mixture was heated for 15 minutes at 96°C and then subjected to 30 cycles of 1 minute at 96°C, 1 minute at 55°C, and 30 seconds at 72°C. The amplified product was hybridized to a set of 43 immobilized oligonucleotides, each corresponding to one of the unique spacer DNA sequences within the DR locus. After hybridization, the membrane was washed twice

Table 2. Distribution and tropism of tuberculous lesions in the lymph nodes and lung lobes in 91 postmortem positive camels with lesions in at least one tissue or organ.

	No (%) of camels with TB lesions			
Tissue	**Total**	**Positive organs or tissue**	**χ^2**	**P-value**
Lymph nodes			22.697‡	*0.002
Parotid	91	13 (14.3%)		
Mandibular	91	15 (16.5%)		
Retropharyngeal	91	17 (18.7%)		
Mediastinal	91	30 (33%)		
Left bronchial	91	17 (18.7%)		
Right bronchial	91	21 (23.1%)		
Mesenteric	91	31 (34.1%)		
Hepatic	91	3 (3.3%)		
Lung lobes			17.901‡	*0.006
Left apical	91	30 (33%)		
Left cardiac	91	27 (29.7%)		
Left diaphragmatic	91	22 (24.2%)		
Right apical	91	25 (27.5%)		
Right cardiac	91	27 (29.7%)		
Right diaphragmatic	91	19 (20.9%)		
Right accessory	91	18 (19.8%)		

*Statistically significant.

‡Chi-square was calculated from the median of pathology score of among tissues examined.

doi:10.1371/journal.pone.0015862.t002

for 10 minutes in 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH2PO4, and 1 mM EDTA[pH 7.7])-0.5% sodium dodecyl sulfate at 60°C and then incubated in 1:4000 diluted streptavidin-peroxidase (Boehringer) for 45 to 60 minutes at 42°C. The membrane was washed twice for 10 minutes in 2× SSPE-0.5% sodium dodecyl sulfate at 42°C and rinsed with 2× SSPE for 5 minutes at room temperature. Hybridizing DNA was detected by the enhanced chemiluminescence method (Amersham) and by exposure to X-ray film (Hyperfilm ECL, Amersham) as specified by the manufacturer.

Data management and analysis

Data were classified, filtered and coded using MS Excel 5, and was transferred to STATA version 8 for statistical analysis. Mean and standard error of the mean were used to summarize pathology scores. Similarly, proportions were used to summarize categorical exposure and outcome measures. Friedman test was used to compare pathology score of tropism of TB lesions among lymph nodes as well as among lung lobes. Bivariate and multivariable logistic regression analyses were used to assess the strength of associations of selected factors and prevalence of camel TB. Effects were reported as statistically significant if p-value was less than 5%. Odds ratio and 95% confidence intervals were used to measure the strength of associations.

Results

Prevalence of camel tuberculosis

On the basis of gross pathology, the prevalence of camel TB was 10% (91/906). Culture positivity was confirmed in 34% (31/91) of the camels with suspicious TB lesions. The result of the association of the different risk factors to the pathology showed that having a good body condition has a protective effect against being positive for TB (Table 1).

Pathology scoring

The distribution of lesions and the severity of the disease were established in the 91 camels with suspicious lesions. The tropism of TB lesions to specific lymph nodes and lung lobes was statistically significant among the lymph nodes ($\chi^2 = 22.697$; $P = 0.002$) and lung lobes ($\chi^2 = 17.901$; $P = 0.006$) (Table 2). Lung lesions were

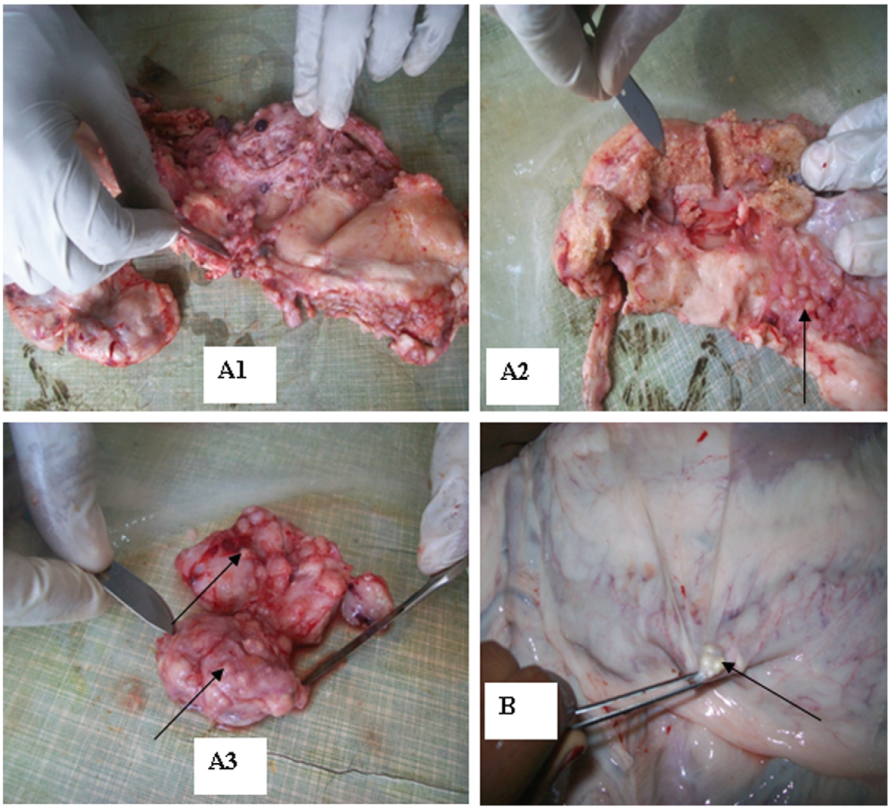

Figure 1. Tuberculous lesions from camels on different organs. (A1) Disseminated and distinct tuberculous lesions in mediastinal parts of the lung. (A2) Tuberculous lesion in mediastinal lymph node and nodules on other parts as indicated by arrows. (A3) Tuberculous lesions in hepatic lymph node. The arrows show that pea-sized lesions throughout the lymph node. (B) Tuberculous lesion in mesenteric lymph nodes as indicated by arrow.
doi:10.1371/journal.pone.0015862.g001

Table 3. Mean pathology and standard error of the mean scoring of the lungs and lymph nodes of camels.

Lung lobes	Mean ± SEM	Lymph nodes	Mean ± SEM
Left apical lobe	0.64±0.11	Parotid	0.27±0.08
Left cardiac lobe	0.63±0.12	Mandibular	0.34±0.09
Left diaphragmatic lobe	0.56±0.11	Retropharyngeal	0.30±0.08
Right apical lobe	0.69±0.13	Mediastinal	0.55±0.15
Right cardiac lobe	0.72±0.13	Left bronchial	0.31±0.08
Right diaphragmatic lobe	0.47±0.11	Right bronchial	0.44±0.09
Right accessory lobe	0.41±0.10	Mesenteric	0.64±0.11

SEM = Standard Error of the Mean.
doi:10.1371/journal.pone.0015862.t003

detected in 43 camels while 78 camels had at least one lesion in their lymph nodes. The lesions appeared more frequent in the apical and cardiac lobes of both lungs than in the diaphragmatic lobes (Table 2). Similarly, the severity was greater in both right apical and cardiac lobes. Regarding lymph nodes, mesenteric lymph nodes were found the most frequently and severely affected of all the lymph nodes (34%) (Table 2, Figure 1).

The mean severity of pathology of camel TB is summarized in Table 3. The mesenteric lymph node constituting the most severely affected lymph node (0.64±0.11; 0.55±0.15) followed by mediastinal lymph node (0.27±0.08).

Mycobacteriology

Growth of mycobacteria was observed in 34% (31/91) of camels with suspicious TB lesion (see Figure 2). Culture positivity was highest (58.8%) in the retropharyngeal lymph node followed by the mesenteric lymph node (35.5%). In contrast, isolation from mandibular and parotid lymph nodes were less frequently mycobacterial culture positive with the positivity of 13.3% and 15.4%, respectively.

Molecular characterization of the isolates

Multiplex PCR. Further *Mycobacterium* genus typing was conducted on the 31 culture isolates from camels. Based on multiplex PCR using the primers of the *M. tuberculosis* complex and *M. avium* complex, 21 isolates gave signal to the genus *Mycobacterium*. Two of these isolates were confirmed to be members of the *M. tuberculosis* complex and none of the isolates were *M. avium* complex (Figure 3).

RD4 deletion typing. The two isolates that showed signal to *M. tuberculosis* complex were subjected to RD4 deletion typing for further differentiation of species and they were confirmed to be *M. bovis* (Figure 4).

Spoligotyping. The two isolates that showed signal with RD4 deletion PCR typing were further characterized using spoligotyping. One of these confirmed to be SB0133 and the other one was a new strain which was not reported previously in *M. bovis* database. The new strain was reported to the global database (http://www.Mbovis.org) and designated as SB1953 (Figure 5). The SB0133 isolate was isolated from camel with generalized and disseminated form of TB.

Discussion

In general, there is scanty information on TB in camels. Nonetheless, there are few reports published on camel TB in Ethiopia as well as in other countries. The prevalence of camel TB recorded by the present study is similar to the report of previous

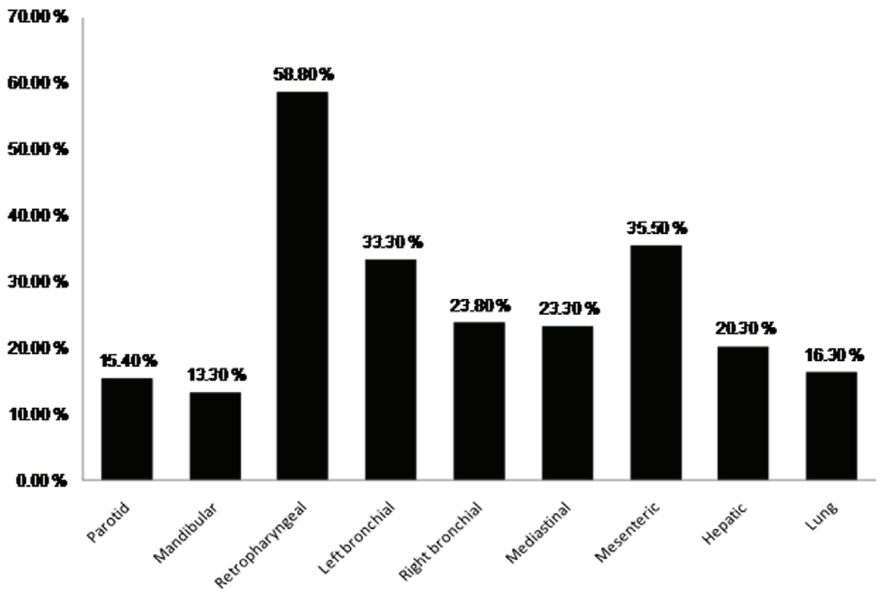

Figure 2. Proportion of mycobacterial culture positivity of the lymph nodes and lungs of TB suspected camels.
doi:10.1371/journal.pone.0015862.g002

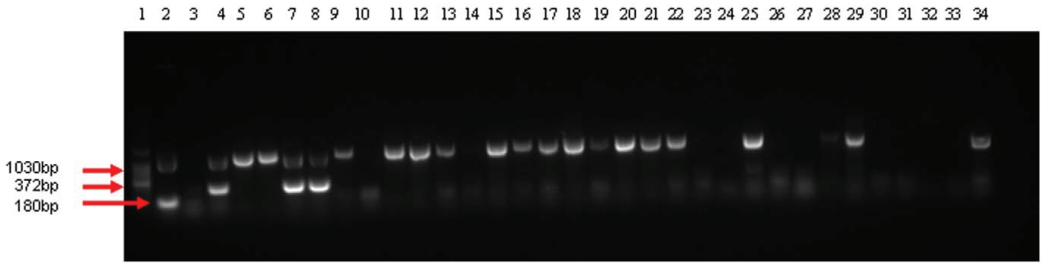

Figure 3. Gel electrophoresis separation of PCR products by multiplex PCR genus typing of mycobacteria isolated from naturally infected camels. Lane 1 = 100bp DNA Ladder; Lane 2 = *Mycobacterium avium* complex (positive control), Lane 3 = Qiagen H_2O (negative control), Lane 4 = *Mycobacterium tuberculosis* complex (positive control), Lanes 5–34 were isolates from individual camels with tuberculous lesions. Lane 7 (sample 63), Lane 8 (sample 62) were positive for *Mycobacterium tuberculosis* complex and Lane 5, 6, 7, 8, 9, 11, 12–13, 15–22, 25, 28, 29, 34 were positive for genus Mycobacterium, Lane 10, 14, 23, 24, 26, 27, 30–33 were negative for genus Mycobacterium.
doi:10.1371/journal.pone.0015862.g003

study in the Afar Region of Ethiopia based on comparative intradermal tuberculin test in camels [23] but it is higher than the report from Dire Dewa Abattoir in camels from eastern Ethiopia [12]. Similarly, it is higher than the prevalence reported in Egypt [6].

The occurrences of TB lesions in camels were relatively higher in the younger and older camels than other age groups. Other researchers have also reported in cattle particularly that older animals are affected by TB [24–27] which could be due to the fact that older animals have weaker immune system. The higher frequency of lesion in younger camels could be due to the less developed immunity [28]. Young camels can also be easily infected with higher doses of mycobacteria via colostrums from infected camel in a similar way, as it occurs in cattle [29]. In connection with this, another report mentioned of vertical transmission of *M. bovis* from an infected dam to her calf through

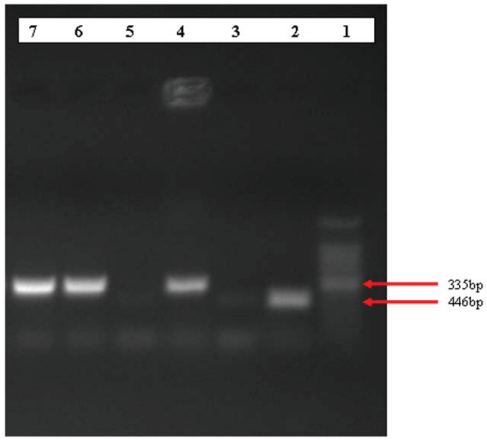

Figure 4. Gel electrophoresis separation of PCR products by RD4 deletion typing of mycobacteria isolated from naturally infected camels. Lane 1 = 100bp DNA ladder, Lane 2 = *M. tuberculosis* positive control, Lane 3 = Qiagen H_2O (negative control), Lane 4 = *M. bovis* positive control, Lane 5–7 were isolates from camel, Lane 6 and 7 were positive for *M. bovis*.
doi:10.1371/journal.pone.0015862.g004

congenital infection in utero [30]. It was observed that lesion was more frequently observed in female camels as compared to male camels. This could be due to the fact that female camels were brought for slaughter at their older age after completion of the reproductive age [26,31].

The distribution, frequency, and severity of lesions recorded in different tissues of camels were similar with the reports of similar studies in grazing cattle in Ethiopia [18,32]. In these studies, the frequency and severity of the lesions were higher in the mesenteric lymph nodes than the thoracic lymph nodes, while in other studies under intensive cattle husbandry lesions were predominant in the respiratory tract and thoracic lymph nodes [14,16].

Tuberculous lesions were subjected to bacteriological culture so as to identify and characterize the causative agents. However, culture positivity of suspicious tissues was 34%, which is lower than what have been reported previously from cattle [18,32]. The lower culture positivity might be related to the non-optimal condition of the culture for NTM which assumed to be the major isolates causing pathology in camel. Regarding culture positivity of each organ, the highest culture positivity was recorded in the retropharyngeal lymph node followed by mesenteric lymph node, which could suggest that oral route could be the main route of infection. In contrast, other authors have reported that culture positivity was higher in lung tissue and thoracic lymph nodes than in the head and mesenteric lymph nodes [14,32,33].

Genus typing of the isolates revealed that out of 21 isolates which showed signals for the genus *Mycobacterium*, only two isolates were *M. bovis* as confirmed by RD4 deletion typing and spoligotyping, while the remaining 18 did not show signal to the *M. tuberculosis* complex, and hence assumed to be members of nontuberculous mycobacteria (NTM). In the present study, the NTM resulted sarcoid-like tuberculous nodules with granulomatous and caseous lesions in lymph nodes, lung and other organs of camel. Previous study reported also the isolation NTM including *M. kansasii* and *M. smegmatis* from tuberculous like lesions in camel causing a similar caseous nodules like those caused by *M. bovis* and *M. tuberculosis* [11]. In Ethiopia, NTM have been isolated from cattle with tuberculous lesions in different regions of the country [34], which indicates their wider geographic distribution and role as a cause of tuberculous lesions in livestock of the country. Therefore, further identification and characterization of these isolates are necessary.

Spoligotyping of the two *M. bovis* isolates revealed a distinct spoligopattern. Referring to the global http://www.Mbovis.org

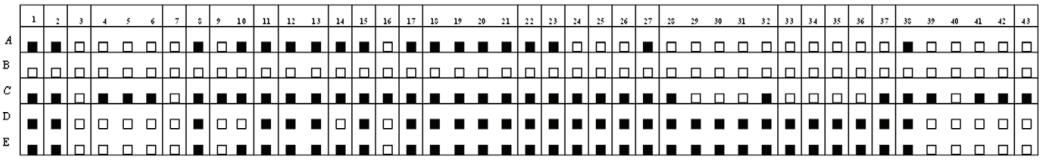

Figure 5. Schematic representation of the spoligotyping patterns of isolates of *Mycobacterium bovis* from camels with tuberculous lesions. A = *M.bovis* SB1176 (positive control); B = Qiagen H_2O (negative control); C = *M. tuberculosis* (positive control); D = sample 63 (SB1953-New strain); E = sample 62 (SB0133). The black rectangles represent positive signals, and the white rectangles indicate negative signals.
doi:10.1371/journal.pone.0015862.g005

database of the spoligopatterns indicated that one of the strains which caused a generalized disseminated TB in camel was SB0133, whereas the other strain was new strain not reported in the database previously. The new strain now has been reported to the database and designated as SB1953. In Ethiopia, a number of studies reported new strains with specific spoligotype pattern in cattle [32,34–36]. The identification of this new *M. bovis* strain from camel TB case of pastoral area of Ethiopia indicates the need for further research in identifying the circulating strains of *M. bovis* in various hosts and their distribution in geographical area of the country. On the other hand, the isolation of SB0133 *M. bovis* strain in present study from camel of pastoral area of Ethiopia inline with the isolation of this strain from cattle of southern Ethiopia [34,36] and pastoral area of Uganda [37] indicates the predominant localization of the strain to pastoral regions of Eastern Africa and possible interspecies transmission of the strain among livestock of pastoral area. In addition, the development of TB lesions to generalized disseminated form of TB in camel affected by SB0133 strain might imply its high pathogenicity in camels.

In conclusion, the present study has shown that the majority of camel TB lesions were caused by NTM; hence, further identification and characterization of these species would be useful towards the efforts made to control TB in camels. The isolation of *M. bovis* strain (SB0133) which is similar to cattle strain in pastoral area of East Africa, implies the existence of potential inter-species transmission of the strain among livestock of pastoral area, warrant further investigation to elucidate its epidemiological significance for public health and control of the disease in the region.

Acknowledgments

The authors are grateful to Addis Ababa Abattoir Enterprise (Akaki) and Metahara Abattoir for their support during sample collection. We also thankful for Dr. Stefan Berg for provision of primers and other reagents for PCR, TB laboratory staffs at Aklilu Lemma Institute of Pathobiology and Faculty of Veterinary Medicine of Addis Ababa University for their support during the laboratory work.

Author Contributions

Conceived and designed the experiments: G. Mamo G. Bayleyegn GA. Performed the experiments: G. Mamo G. Bayleyegn GA. Analyzed the data: G. Mamo G. Bayleyegn GA G. Medhin. Contributed reagents/materials/analysis tools: G. Mamo GA ML FA. Wrote the paper: G. Mamo G. Bayleyegn. Revised the final version of the paper for publication: G. Mamo G. Bayleyegn TST FA GA ML G. Medhin G. Bjune.

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Hindawi Publishing Corporation
Veterinary Medicine International
Volume 2012, Article ID 869146, 8 pages
doi:10.1155/2012/869146

Research Article

Tuberculosis in Goats and Sheep in Afar Pastoral Region of Ethiopia and Isolation of *Mycobacterium tuberculosis* from Goat

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Received 3 February 2012; Accepted 21 May 2012

Academic Editor: Michael D. Welsh

A cross sectional study was conducted on 2231 small ruminants in four districts of the Afar Pastoral Region of Ethiopia to investigate the epidemiology of tuberculosis in goats and sheep using comparative intradermal tuberculin skin test, postmortem examination, mycobacteriological culture and molecular typing methods. The overall animal prevalence of TB in small ruminants was 0.5% (95% CI: 0.2%–0.7%) at ≥4 mm and 3.8% (95% CI: 3%–4.7%) at cutoff ≥2 mm. The herd prevalence was 20% (95% CI: 12–28%) and 47% (95% CI: 37–56%) at ≥4 mm and ≥2 mm cut-off points, respectively. The overall animal prevalence of Mycobacterium avium complex infection was 2.8% (95% CI: 2.1–3.5%) and 6.8% (95% CI: 5.8–7.9%) at ≥4 mm and ≥2 mm cut-off points, respectively. Mycobacteriological culture and molecular characterization of isolates from tissue lesions of tuberculin reactor goats resulted in isolation of *Mycobacterium tuberculosis* (SIT149) and non-tuberculosis mycobacteria as causative agents of tuberculosis and tuberculosis-like diseases in goats, respectively. The isolation of *Mycobacterium tuberculosis* in goat suggests a potential transmission of the causative agent from human and warrants further investigation in the role of small ruminants in epidemiology of human tuberculosis in the region.

1. Background

Ethiopia has one of the largest resources of goats and sheep among African countries, with an estimated number of 21.9 million goats and 25.9 million sheep [1]. Goats and sheep contribute significantly to the economy and food security of the poor farmers in the country. About 73% of the national goat population and 25% of the sheep population are found in the lowland pastoral areas of the country [2]. In pastoralist area, goats and sheep are mainly utilized for milk and meat production and generate income to the owner. In spite of the large population and potential use of small ruminants, the production system is affected by feed shortage, poor genetic makeup of the animals, and wide spread occurrence of livestock diseases such as tuberculosis which has both economic and public health significance to the communities.

TB in goat and sheep is caused by members of *Mycobacterium tuberculosis* complex predominantly by *Mycobacterium bovis* and *Mycobacterium caprae* [3–14] and few caused by *Mycobacterium tuberculosis* [15, 16]. Epidemiological studies indicated that tuberculosis in goat and sheep has a wide global distribution and has been reported in various countries of the world including New Zealand, Sudan, Spain, Nigeria, the United Kingdom, Italy, Algeria, Ethiopia [3–17]. In Ethiopia, bovine TB has been known to be endemic in cattle; however, the status of TB in goats and sheep has not been well studied in spite of their close contact with cattle. Few studies carried out so far in central highland Ethiopia

indicated the existence of TB in small ruminants with low level of prevalence (4.2%) based on abattoir examination results [14] and 3.1% using single intradermal tuberculin test [17]. In lowland pastoral area where the large population of goats and sheep exists, the status of the disease is unknown.

Livestock in pastoralist region is major source of food and income, in addition, possession of livestock provides a measure of social status in the pastoral communities. In pastoralist communities of Ethiopia including the Afar, pastoralists' habit of consumption of raw animal product particularly milk is common and the pastoralists have close physical contact with their animals. Afar pastoralists consume both goat and sheep milk very commonly, and to protect these small ruminants from predators, the pastoralists keep these animals in very close proximity to their houses. These conditions are potential risk factors for transmission of zoonotic diseases such as TB of animal origin to human or vice versa. Goats and sheep have also common watering and grazing points with cattle that might favor the transmission of mycobacterial species among these domestic animals. Previous studies in cattle and camel of pastoral regions indicated the endemic nature of TB in the regions [18–22]. Therefore, the present study was designed to investigate the epidemiology of TB in goats and sheep and characterizes the causative agents in the Afar Pastoral Region of Ethiopia.

2. Material and Methods

2.1. Study Area. The study was conducted in four districts, namely, Amibara, Dubti, Afambo, and Chifra districts of Afar Pastoral Region. The Afar Pastoral Region is located in northeast of Ethiopia between 39°34′ and 42°28′ E longitude and 8°49′ and 14°30′ N latitude. The region shares common international boundaries with Eritrea in the northeast and Djibouti in the east, and it is characterized by an arid and semiarid climate with low and erratic rainfall [23].

In the Afar Region, there are about 4,268,000 goats and 2,464,000 sheep which are managed under pastoral and agropastoral production system [24]. Afar pastoralists own different species of domestic animals, and these animals share common watering points and grazing sites. Small ruminants usually graze/browse near their villages, while cattle and camel might travel a long distance in search of grass and browsing trees. The watering points of small ruminants are commonly shared with cattle and camel creating a close interspecies interaction among these domestic animals, and this might increases the risk of transmission of mycobacteria from cattle or camels to small ruminants or vice versa.

2.2. Study Design. A cross-sectional study was conducted in the four districts of the Afar Pastoral Region and a total of 14 subdistricts and 21 villages were included in the study based on the inclusion criteria (accessibility, security, and willingness of the pastoralists to participate in the research). All villages in each subdistrict were included after obtaining the elder clan leaders' consent to participate in the study. In this study, goat and sheep kept by an owner and his close relatives in which case if the animals share common grazing sites, watering points, and night shelter, they were considered as a herd to calculate the herd prevalence. A total of 103 flocks (herds) of small ruminants were tested by CIDT test.

2.3. Study Animals. For the CIDT test, small ruminants above the age of six months having no clinical symptom of any disease were included. Study animal-related information on each tested sheep and goat (such as sex, age, body condition score, lactation and reproductive status, and parity number) was collected and recorded at the time of the test. Each animal was dewormed with antihelmintic drug after testing. A total of 2231 small ruminants (1884 goat and 347 sheep) were tested using CIDT.

2.4. Comparative Intradermal Tuberculin Skin Test (CIDT). CIDT test was carried out by injecting both bovine purified protein derivative (PPD) and avian PPD (observe bovine and avian tuberculin, AsureQuality Company, Mt. Wellington, Auckland, New Zealand). Two sites on the skin of the midneck of the animal, 12 cm apart, were shaved, and skin thickness was measured with a caliper. One site was injected with an aliquot of 0.1 mL of 2,500-IU/mL bovine PPD into the dermis, and the other was similarly injected with 0.1 mL of 2,500-IU/mL avian PPD. After 72 h, the skin thickness at the injection site was measured and recorded. Results were interpreted according to the recommendations of the Office International des Epizooties [25] at ≥4 mm cutoff and also at ≥2 mm cutoff [26]. Thus, at cutoff ≥4 mm, if the increase in skin thickness at the injection site for bovine PPD (PPD-B) was greater than the increase in skin thickness at the injection site for avian PPD (PPD-A) and PPD-B minus PPD-A was less than 2 mm, between 2 and 4 mm, or 4 mm and above, the animal was classified as negative, doubtful, or positive reactor based on CIDT test, respectively. At cutoff ≥2 mm, if the difference between PPD-B and PPD-A was greater or equal to 2 mm, the animal was considered as positive, while if the difference is less than 2 mm, the animal was considered as negative. When the change in skin thickness was greater at PPD-A injection site, the animal was considered positive for mycobacteria species other than *Mycobacterium tuberculosis* complex. A flock (herd) was considered as positive if it had at least one tuberculin reactor animal.

2.5. Body Condition Scoring. The body condition scoring for goat and sheep was carried out using the guidelines established by Langston University and ESGIP guidelines for body condition scoring [27, 28]. Accordingly, on the basis of observation of anatomical parts such as vertebral column, ribs, and spines, the study animals were classified as lean (score 1 to 2), medium (3 to 4), or fat (greater than 5).

Age determination was carried out based on the dentition according to Vatta and his coworkers [29] and adopted ESGPIP guideline for estimation of age of sheep and goat [30].

2.6. Postmortem Examination. Tissues with suspicious lesions from five slaughtered tuberculin reactor goats were

collected aseptically from the lung lobes (left apical, left diaphragmatic, right apical, right cardiac, right diaphragmatic, and right accessory), lymph nodes of the head (retropharyngeal and mandibular), lymph nodes of lungs (mediastinal and bronchial), and mesenteric lymph nodes. Data were collected on the presence, size, and distribution of visible lesions in each carcass. Samples from tissues containing visible lesions were collected and placed into sterile universal bottles containing 5 mL of 0.9% saline solution(pH 7.2) and kept at −20°C at Semera Regional Animal Health Laboratory until they were transported to ALIPB laboratory under cold chain for isolation of the causative agents.

2.7. Isolation of Mycobacteria from Tissue Samples. Isolation of mycobacteria from tissues was done in accordance with OIE protocols [31]. Briefly, the specimens were sectioned into pieces using sterile blades and then homogenized by pestle and mortar. The homogenate was decontaminated by adding an equal volume of 4% NaOH followed by centrifugation at 1000 g for 15 minutes. The supernatant was discarded, while the sediment was neutralized by 1% (0.1N) HCl using phenol red as an indicator. Neutralization was achieved when the colour of the solution changed from purple to yellow. Thereafter, 0.1 mL of suspension from each sample was spread onto a slope of Löwenstein-Jensen (LJ) medium. Duplicates of LJ media were used; one enriched with sodium pyruvate, while the other was enriched with glycerol. Cultures were incubated at 37°C in a slant position for one week and in upright position for 11 weeks with weekly observation for mycobacterial growth. Whenever, colonies were seen, subculturing and Ziehl-Neelsen staining were performed to confirm the presence of acid fast bacilli. Positive colonies were preserved with freezing media, and some portions of the colonies were heat-killed in water bath at 80°C for 45 minutes. The frozen and heat killed isolates were stored at −20°C for future mycobacteriology and further molecular typing analysis.

2.8. Molecular Characterization of Mycobacterial Isolates. Mycobacterium genus typing was conducted as described previously [32], and spoligotyping of *Mycobacterium tuberculosis* complex isolate from goat was performed as previously described by Kamerbeek and coauthors [33]. Both methods were described in detail in previous publication [22].

2.9. Data Management and Analysis. Data were classified, filtered, coded using EpiData software and Microsoft Excel sheet, and was transferred and analyzed using STATA version 11 (Stata Corp., Collage station, TX). Pearson chi-square was used to evaluate the statistical significance of the associations of different categorical variables with skin test results. Bivariate and multivariable logistic regression analyses were performed to quantify crude and adjusted effects of prespecified risk factors on tuberculin reactivity. P values less than 5% were considered statistically significant. In cases of estimating the effect of different risk factors in terms of OR with corresponding 95% confidence interval, statistically significance was assumed if the confidence interval did not include one among its values.

3. Results

3.1. Animal Prevalence. On the basis of CIDT test, the animal prevalence of TB was 0.5% (10/2231) at a cutoff ≥4 mm and 3.8% (86/2231) at a cutoff ≥ 2 mm. At ≥2 mm cut-off point, there were significant differences in proportions of reactors among the four districts (χ^2 = 26.385, P = 0.000), between sheep and goat (χ^2 = 6.46, P = 0.011) and between pregnant and nonpregnant females (χ^2 = 5.342, P = 0.021) (Table 1). Multivariable logistic regression analysis at ≥2 mm cut-off point showed that older small ruminants (5 years and above) had 13 times the odds of being tuberculin reactors compared with age category less than 2 years old (adjusted OR = 13.79; CI: 2.22–85.55). Female small ruminants with parity number greater than 4 had 0.05 odds of being bovine tuberculin positivity in relative to those with less than 2 parity numbers (adjusted OR = 0.05; CI: 0.01–0.31) (Table 2). At ≥4 mm cut-off points, there was no statistical significance difference in the proportion of bovine tuberculin positivity between groups in relation to the different variables considered.

3.2. Herd Prevalence. The herd prevalence was 20% (95% CI: 12–28%) and 47% (95% CI: 37–56%) at ≥4 mm and ≥2 mm cut-off points, respectively. In multivariable logistic regression analysis, no significant association was found in herd positivity between groups in relation to district of origin, herd size category, and production system at ≥2 mm cut-off point (Table 3).

3.3. Prevalence of Mycobacterium Avium Complex Infection. According to the CIDT test result of the avian tuberculin skin reaction, the overall animal prevalence of *Mycobacterium avium* complex infection was 2.8% (95% CI: 2.1–3.5%) and 6.8% (95% CI: 5.8–7.9%) at ≥4 mm and ≥2 mm cut-off points, respectively.

3.4. Postmortem Lesions in Tuberculin Reactor Goats. Five goats which were positive to bovine TB in CIDT test at cutoff ≥ 4 mm were purchased, slaughtered, and investigated for gross tuberculous lesions. Tuberculous lesions were detected in different organs (left diaphragmatic lung, retropharyngeal lymph node, parotid lymph node, right bronchial lymph node, mesenteric lymph node, intestinal wall, and mesentery). Two of them had partially disseminated TB lesions which involved lung, intestine and the lymph nodes of thoracic and abdominal cavities. Upon incision of the lung, lesions showed a yellowish caseous material indicating a characteristic of tuberculous lesion (Figure 1). In mesentery and mesenteric lymph nodes, greenish discharge was observed in the lesions.

3.5. Molecular Typing of the Isolates from Goats. All tissue samples obtained from slaughtered tuberculin reactor goats were positive for mycobacterial growth on LJ culture medium. Further molecular characterization indicated that

Table 1: Association of different risk factors to skin test positivity at ≥2 mm cut-off point for small ruminant tuberculosis in Afar Pastoral Region of Ethiopia.

Variables	Number of animals examined	Number of positive (%)	χ^2	P value
Districts				
Chifra	396	20 (5.05)		
Dubti	237	22 (9.28)	26.385	0.000*
Afambo	117	5 (4.27)		
Amibara	1481	39 (2.63)		
Species				
Ovine	347	5 (1.44)	6.460	0.011*
Caprine	1884	81 (4.3)		
Herd size				
≤25	617	32 (5.19)		
$11 < X \leq 50$	851	32 (3.76)	4.915	0.086
>50	763	22 (2.88)		
Sex				
Male	206	11 (5.34)	1.351	0.245
Female	2025	75 (3.70)		
Age#				
≤2	594	18 (3.03)		
$2 < X < 5$	779	36 (4.62)	2.361	0.307
$X \geq 5$	858	32 (3.73)		
BCS				
Poor	376	13 (3.46)		
Good	1116	48 (4.30)	1.204	0.548
Fat	739	25 (3.38)		
Production system				
Pastoral	2051	80 (3.90)	0.144	0.705
Agropastoral	180	6 (3.33)		
Lactation status				
Kid/lamb	57	2 (3.51)		
Lactating	760	19 (2.50)	1.255	0.534
Nonlactating	354	13 (3.67)		
Reproductive status				
Nonpregnant	857	19 (2.22)	5.342	0.021*
Pregnant	314	15 (4.78)		
Parity number				
<2	260	10 (3.85)		
$2 \leq X < 4$	304	13 (4.28)	4.415	0.110
$X \geq 4$	324	5 (1.54)		

#A given age range includes its lower bound and excludes its upper bound; BCS: body condition score; *statistically significant.

one of the isolates was human type *Mycobacterium tuberculosis* (SIT149) from goat specimen (Figure 2), and the others were nontuberculosis mycobacteria species. The goat with SIT149 isolate was strong reactor to bovine tuberculin test with high skin induration difference (PPD-B minus PPD-A = 10 mm), and the postmortem examination result showed typical tuberculin lesions in lung, bronchial lymph nodes, caudal mediastinal lymph node, and also on mesenteric lymph nodes while goats from which nontuberculosis mycobacteria species were isolated have showed indurations of skin at both avium and bovine tuberculin injection site. In addition, the pathological lesions observed in postmortem examination were localized in retropharyngeal lymph nodes and mesenteric lymph nodes.

4. Discussion

Little information is available on TB in small ruminants in Ethiopia even though bovine TB is known to be endemic in cattle of Ethiopia [34]. In this study, a prevalence of 0.5% at ≥4 mm cut-off and 3.8% at ≥2 mm cut-off point was recorded in small ruminants in four districts of Afar Pastoral

Table 2: Multivariable logistic regression analysis of tuberculin reactors with various host-related risk factors at ≥2 mm cut-off point.

Variables	Number of animals examined	Number (%) of positive in CIDT	Crude odds ratio (95% CI)	Adjusted odds ratio (95% CI)
Districts				
Chifra	396	20 (5.05)	1	1
Dubti	237	22 (9.28)	1.92 (1.03–3.61)*	—
Afambo	117	5 (4.27)	0.84 (0.31–2.29)	—
Amibara	1481	39 (2.63)	0.51 (0.29–0.88)*	0.17 (0.05–0.55)*
Species				
Ovine	347	5 (1.44)	1	1
Caprine	1884	81 (4.3)	3.07 (1.24–7.64)*	2.05 (0.42–9.94)
Herd size				
≤25	617	32 (5.19)	1	1
$11 < X \leq 50$	851	32 (3.76)	0.71 (0.43–1.18)	1.84 (0.52–6.45)
>50	763	22 (2.88)	0.54 (0.31–0.94)*	0.44 (0.14–1.34)
Sex				
Male	206	11 (5.34)	1	1
Female	2025	75 (3.70)	0.68 (0.36–1.31)	0.25 (0.04–1.74)
Age				
≤2	594	18 (3.03)	1	1
$2 < X < 5$	779	36 (4.62)	1.55 (0.87–2.76)	2.16 (0.47–9.89)
$X \geq 5$	858	32 (3.73)	1.24 (0.69–2.23)	13.79 (2.22–85.55)*
BCS				
Poor	376	13 (3.46)	1	1
Good	1116	48 (4.30)	1.25 (0.67–2.34)	1.90 (0.61–5.88)
Fat	739	25 (3.38)	0.98 (0.49–1.93)	0.75 (0.17–3.28)
Production system				
Pastoral	2051	80 (3.90)	1	1
Agropastoral	180	6 (3.33)	1.18 (0.51–2.74)	—
Lactation status				
Kid/lamb	57	2 (3.51)	1	1
Lactating	760	19 (2.50)	1.05 (0.23–4.77)	0.50(0.06–4.08)
Nonlactating	354	13 (3.67)	0.71 (0.16–3.11)	0.82(0.12–5.79)
Reproductive status				
Nonpregnant	857	19 (2.22)	1	1
Pregnant	314	15 (4.78)	2.21 (1.11–4.41)*	3.43(0.72–16.33)
Parity number				
<2	260	10 (3.85)	1	1
$2 \leq X < 4$	304	13 (4.28)	1.12 (0.48–2.59)	0.38 (0.09–1.65)
$X \geq 4$	324	5 (1.54)	0.39 (0.13–1.16)	0.05 (0.01–0.31)*

CI: confidence interval, BCS: body condition scoring, *statistically significant.

Region of northeastern Ethiopia. The result was in agreement with that of Hiko and Agga [14] who reported 4.2% in goats slaughtered at Mdjo abattoir and with a report by Tafesse and coauthors [17] who recorded a prevalence of 3.1% in goat with single intradermal tuberculin skin test. A recent study carried out on goats and sheep of central Ethiopia using CIDT also showed a low prevalence of tuberculosis (0.41% at 2 mm cut-off point) [16] which might suggests an overall low prevalence of TB in small ruminants in the country. However, our result was different from the result of a previous study done in Hamer pastoral district of southern Ethiopia, which indicated the absence of the disease in 186 goats using CIDT test [21]. This difference might be related, the difference in geographical location of the two studies in which the epidemiology of the disease might vary between these areas.

The proportion of positive reactors was significantly higher in Dubti district than the other districts which might be related to the husbandry system where small ruminants had higher interaction with cattle in Dubti districts than

Table 3: Multivariable logistic regression analysis of herd TB positivity with selected risk factors at ≥2 mm cut-off point.

Variables	Number of herds examined	Number of positive herds (%)	Crude odds ratio (95% CI)	Adjusted odds ratio (95% CI)
Districts				
Chifra	18	10 (55.6)	1	1
Dubti	13	9 (69.2)	1.8 (0.40–8.07)	2.59 (0.49–13.73)
Afambo	6	2 (33.3)	0.40 (0.06–2.77)	2.70 (0.06–114.64)
Amibara	66	27 (40.9)	0.55 (0.19–1.58)	0.45 (0.14–1.39)
Herd size				
≤25	52	19 (36.5)	1	1
$11 < X \leq 50$	34	19 (55.9)	2.2 (0.91–5.31)	3.23 (1.21–8.60)
>50	17	10 (58.8)	2.48 (0.81–7.59)	2.48 (0.76–8.09)
Production system				
Agropastoral	8	3 (37.5)	1	1
Pastoral	95	45 (47.4)	1.5 (0.34–6.64)	6.88 (0.28–170.32)

CI: confidence interval.

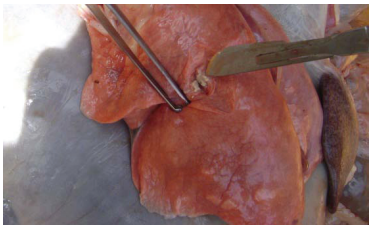

Figure 1: Tuberculous lesion from goat lung caused by *Mycobacterium tuberculosis*.

the other districts, which can favor a potential transmission of mycobacterial species between cattle and goat. Older goat and sheep showed higher proportion of positivity in tuberculin test results which might be related to the fact that older animals have longer duration and repeated chance of exposure to mycobacterial infection with their age. Similar results have been reported by other researchers in cattle [34, 35]. Female animals with more parity number showed higher proportion of positivity in tuberculin test results than in those with lower parity number. This might be related to the age of the animals as animals with high parity number were older in their age which increases their chance of exposure to mycobacterial infection in their longer life time.

Mycobacteriological culture of the tissue lesions from the five tuberculin reactors goats had resulted in the isolation of *Mycobacterium tuberculosis* and nontuberculosis mycobacteria species. In this study, *Mycobacterium tuberculosis* strain SIT149 was isolated from a goat suggesting the possibility of its transmission from human to goat. Similar strain has been isolated in camel from pastoral region in south east of Ethiopia [36]. The SIT149 strain of *Mycobacterium tuberculosis* is a dominant strain in Ethiopia [37], and it was a common isolate in human pulmonary TB patients from the same Afar Pastoral Region indicating that the isolate has been circulating in the area. Afar pastoralists have close contact with goats and sheep and often keep young goats and sheep in their house at night which might be a potential factor for transmission from human patient to animals. Previous studies in cattle of Ethiopia demonstrated that *Mycobacterium tuberculosis* was commonly isolated from tuberculous lesions of cattle in different regions of Ethiopia [38, 39].

In sheep, we observed 1.44% prevalence of TB at 2 mm cut-off point and no at 4 mm cut-off point. The result was in agreement with previous studies where sheep TB has been reported both with tuberculin skin test and postmortem examination results [3, 4, 9, 10, 12, 16].

In conclusion, this study revealed a moderately low prevalence of TB in goats and sheep of Afar Pastoral Region of Ethiopia. *Mycobacterium tuberculosis* and nontuberculosis mycobacteria were isolated as causative agents of TB in goats of the region. The isolation of the *Mycobacterium tuberculosis* in goat indicates the need for further studies to understand the interspecies transmission dynamics of *Mycobacterium tuberculosis* and the role of small ruminants in the epidemiology of human tuberculosis in pastoralist setting where potential epidemiological risk factors for infection and transmission between livestock and human exist. In addition, the identification of nontuberculosis mycobacteria from tuberculous lesions in goats indicates their importance in the epidemiology of small ruminant TB and further research is needed to identify the species and their public health significance for the pastoralist communities of the region. In general, similar to the previous studies carried out in cattle and camel of pastoral regions of Ethiopia [18, 20–22, 36] which have indicated the endemic nature of tuberculosis in these species, the result of this study also indicated the importance of tuberculosis in small ruminants of Afar Pastoral Region which further emphasizes the need to design a feasible national TB control strategy in livestock of the country.

Authors' Contribution

G. M. Kassa contributed to the design of the study, participated in collection, analysis, and interpretation of data, drafted and revised the paper. F. Abebe contributed to the design of the study, interpretation of data, and critically

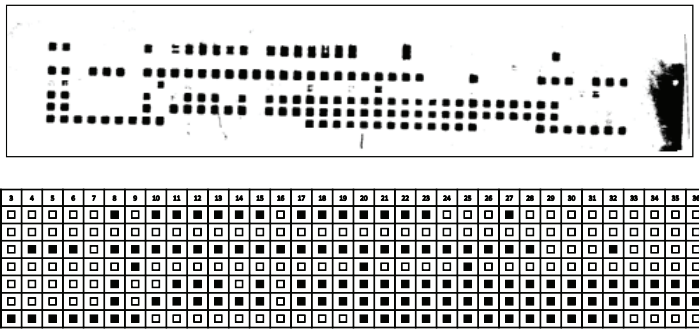

Figure 2: Scanned autorad and schematic representation showing spoligotyping pattern of isolate from the goat with tuberculous lesion caused by *M. tuberculosis*. A: *M. bovis* SB1176 (positive control); B: Qiagen H_2O (negative control); C: M. tuberculosis (positive control); D–F: sample from other animals, G: SIT149 (isolate from goat). The black rectangles represent presence of spacers, and the white rectangles indicate absence of spacers.

revised the paper. Y. Worku and M. Legesse contributed to study design, data collection, data analysis, and interpretation and critically revised the paper. G. Medhin participated in study design, data analysis, and interpretation and critically revised the paper. G. Bjune involved in study design and critically revised the paper. G. Ameni contributed to study design, data collection, data analysis, and interpretation and critically revised the paper. All authors read and approved the final paper.

Acknowledgments

The authors are grateful to Afar pastoralist communities and Afar Pastoral Regional State for willing to participate in the study and supporting the research. The authors acknowledge the Aklilu Lemma Institute of Pathobiology of Addis Ababa University, Institute of Health and Society Department of Community Medicine of University of Oslo, and Faculty of Veterinary Medicine of Addis Ababa University for the overall support of the study. They also thank TB laboratory staff at ALIPB for field and laboratory work. This study was financially supported by the Norwegian Program for Development Research and Education (NUFU PRO-2007/10198).

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Vol. 5(6), pp. 171-179, June 2013
DOI 10.5897/JVMAH2013.0204

http://www.academicjournals.org/JVMAH

Journal of Veterinary Medicine and Animal Health

Full Length Research Paper

Bovine tuberculosis and its associated risk factors in pastoral and agro-pastoral cattle herds of Afar Region, Northeast Ethiopia

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Accepted 15 May, 2013

Bovine tuberculosis (BTB) has a potential public health risk and economic impact in pastoralist community whose livelihood depends on their livestock. A cross-sectional study was carried out from September, 2008 to June, 2011 on 1087 cattle under pastoral and agro-pastoral production system in four districts of Afar Pastoral Region of Ethiopia using comparative intradermal tuberculin skin test to estimate the prevalence of BTB and assess the associated risk factors for infection. The individual animal prevalence of BTB in cattle of Afar pastoralists was 11% (95% confidence interval (CI): 9 to 13%) with ≥ 4 mm cut-off and 18% (95% CI: 16% to 21%) with ≥2 mm cut-off. The herd prevalence was 44% (95% CI: 36 to 51%) and 56% (95% CI: 48 to 63%) at ≥4 and ≥2 mm cut-off points, respectively. In bivariate analysis, the prevalence was significantly associated with study districts, herd size, sex and age, and in multivariable logistic regression analysis, the statistical significance was maintained with study district, age and herd size of the cattle. In conclusion, the present study revealed a moderately high prevalence of BTB in Afar Pastoral Region of Ethiopia and further investigation is recommended to assess the zoonotic significance of the disease to the pastoralist communities of the region.

Key words: Bovine tuberculosis, prevalence, risk factors, comparative intradermal tuberculin test, Afar pastoral region, Ethiopia.

INTRODUCTION

Bovine tuberculosis (BTB) is a chronic, granulomatous mycobacterial infectious disease caused mainly by *Mycobacterium bovis*, which is a member of *Mycobacterium tuberculosis* complex. BTB is a zoonotic disease with a potential health risk to human and has economic significance to livestock sub-sector (Ayele et al., 2004). Though BTB is controlled in developed countries through test-and-slaughter method, the disease poses a significant problem to the economy of the livestock sub-sector and remains a potential public health

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threat in developing countries where controlling programs are lacking. In Africa, approximately 85% of cattle and 82% of human population lives in areas where BTB is partly or not controlled at all in animals (Cosivi et al., 1998) and consumption of raw animal product is a common practice in rural and pastoralist communities of the continent, which creates a potential risk for zoonotic transmission of *M. bovis* (Daborn et al., 1996).

Ethiopia possesses the largest cattle population in Africa with the total of about 51 million of cattle (CSA, 2010). The livestock sub-sector in general contributes about 45% to the gross domestic product of the country's agriculture based-economy (Behnke, 2010). In addition, the sector plays a crucial role in livelihood of the pastoralist communities, who own 42% of the country's livestock in the lowland arid and semi arid regions. In Eastern Africa, Ethiopia has the largest pastoralist population (7 to 8 million) which depends on livestock for their livelihood (Markakis, 2004). The main feature of pastoralist's way of life is that they move from place to place in search of water and pasture for their livestock.

In Ethiopia, BTB is known to be endemic with prevalence ranging from 3.4 to 50% depending on husbandry method, with extensive rural setting showing low prevalence as compared to intensive dairy farms (Ameni et al., 2007; Berg et al., 2009). In spite of large population of livestock, very few studies were carried out in pastoral area of Ethiopia in which prevalence of 0.8% BTB in cattle of Hamer pastoral district (Tschoop et al., 2010) and prevalence of 5.5% of BTB in cattle of Borena pastoral area (Gumi et al., 2011) were reported. However, so far there is no report of BTB in cattle of Afar Pastoral Region of Northeast Ethiopia.

The Afar pastoral communities of Ethiopia are characterized by owing large numbers of livestock with diversity of species of animal. The consumption of raw animal products such as milk and very close physical contact creates a significant risk for transmission of zoonotic diseases like BTB. In addition, the existing epidemiological setting in Afar Pastoral Region is characterized by the presence of large herds of cattle, interspecies mixing of herds of animals at watering point, grazing area, at night in the village and the existence of climatic stress factors in the pastoral regions could suggest the existence of a potential risk factors for infection and transmission of diseases such as BTB in the livestock and pastoral communities of the region. Despite the large livestock population and existence of potential risk factors in the pastoral region, the epidemiology of BTB in the herds of cattle owned by pastoralist has not been well investigated so far.

The present study, therefore, was designed to investigate the epidemiology of BTB and assess the associated risk factors in the Afar Pastoral Region of Ethiopia.

MATERIALS AND METHODS

Study area

The study was conducted from September, 2008 to June, 2011 in four districts namely (Amibara, Dubti, Afambo and Chifra districts) of Afar Pastoral Region. The Afar Pastoral Region is located in northeast of Ethiopia between 39° 34' to 42° 28'E longitude and 8° 49' to 14° 30' N latitude (Figure 1). The region shares common international boundaries with Eritrea in the north-east and Djibouti in the east, and it is characterized by an arid and semi-arid climate with low and erratic rainfall. Rainfall is bi-modal throughout the region, with a mean annual rainfall below 500 mm in the semi-arid western escarpments and decreasing to 150 mm in the arid zones to the east. The altitude of the Region ranges from 120 m below sea level in Danakil depression to 1500 m above sea level. Temperatures vary from 20°C in higher elevations to 48°C in lower elevations. The human population of Afar region is 1.5 million in which the majority are pastoralists who largely depend on livestock production for their livelihood (Afar National Regional State (ANRS), 2010).

There are about 1.9 million Afar breed cattle in Afar Region, of which 90% of the cattle are managed under pastoral production system and the rest 10% in agro-pastoral production system (ANRS, 2010). The four districts were selected based on the cattle population, accessibility of their sub districts and presence of potential risk factors. Because of the presence of large pasture land and rivers in the districts, animals from different districts migrate to river banks and vast pasture lands where intermixing of different species (cattle, camel, goat and sheep) and herds of livestock occur. In Amibara and Dubti districts, there are large state-owned and private cotton farms, which after harvesting, become grazing sites where large number of herds of different species of livestock (cattle, camel and small ruminants) from various districts congregate to graze on the leftovers of the harvest, creating a potential risk factor for interspecies (cattle, camel, goat and sheep) and interherd disease transmission. Majority of the grazing land and watering points in Amibara district are shared by wild animals (including oryx, warthog, gazelle and zebra) from the Awash National Park. It was very common to observe cattle grazing in close proximity with wild animals in the pasture land of Amibara district. The sites selected in Amibara, Chifra and Dubti districts were pastoral and that of Afambo were mainly agro-pastoralist in their production system.

Study design

A cross sectional study was conducted in the four districts of Afar Pastoral Region and a total of 17 sub districts were included in the study based on the inclusion criteria (accessibility, security, and willingness of the pastoralists to participate in the research). All settlements (villages) in each sub district were included after obtaining the elder's consent to participate in the study. In our study, cattle owned by one owner and/or his close relatives, in which the animals shared common grazing sites, watering points, kept at night in common site and move together during migration, were considered as a herd to calculate the herd prevalence. In settlements which had super-herd, larger herd composed 500 to 600 animals, herd selection was made proportionally to represent each cluster in the super-herd. A total of 180 herds were tested and the final analysis was carried out on 171 herds, the rest 9 (5%) were drop-outs in which they were not available for reading after 72 h of tuberculin injection. In each herd, individual cattle were selected randomly after recording all the animals in the herd.

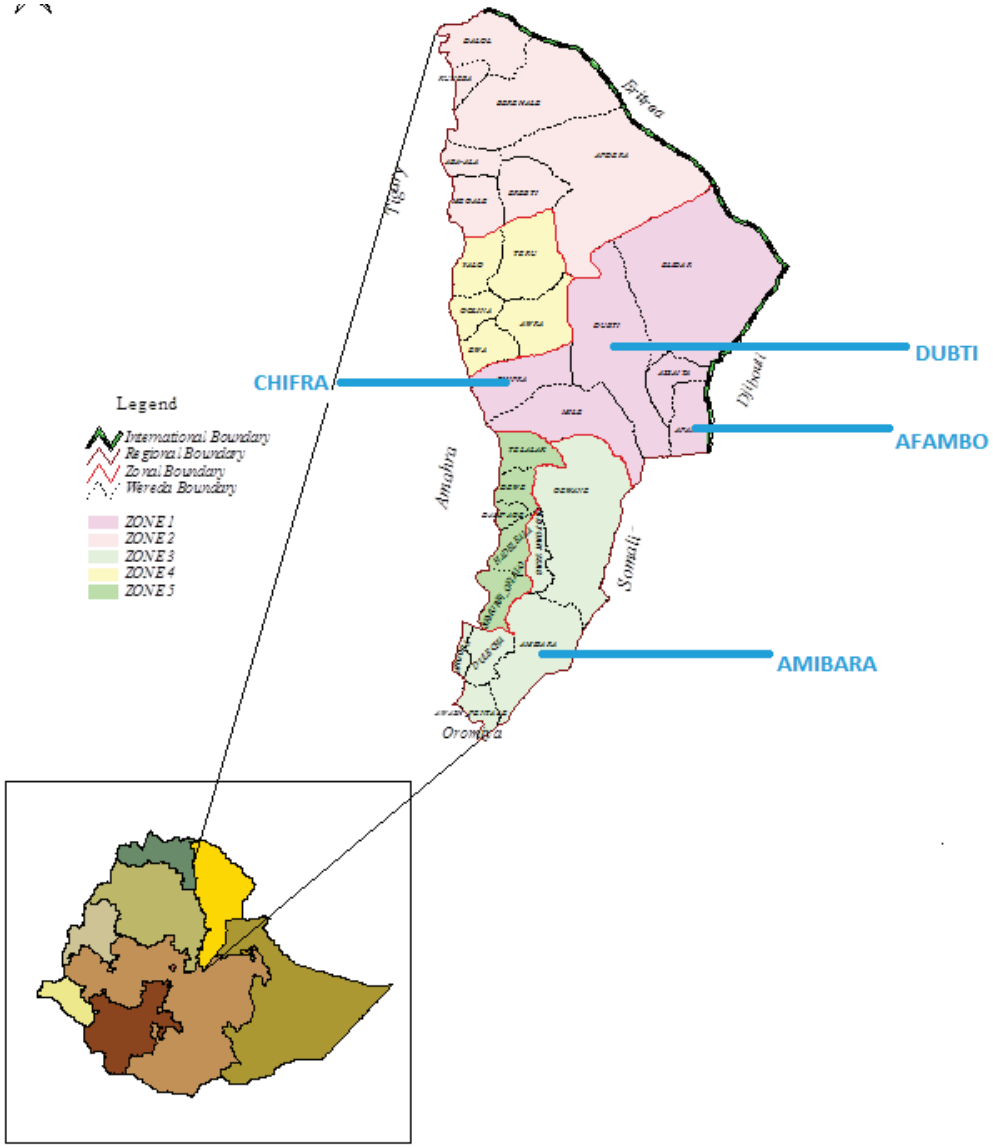

Figure 1. Map of Afar Pastoral Region indicating the study districts.

Study animals

For the Comparative intradermal tuberculin skin test (CIDT), cattle above the age of six months having no clinical symptom of any disease were included. Study animal related information on each tested cattle (such as sex, age, body condition score, lactation and reproductive status, parity number (number of calving)) were collected and recorded at the time of the test. All the cattle in this study were Afar (Danakil) breed of cattle which are categorized under Sanga breed group. Each animal was de-wormed with antihelmintic drug after testing and collecting fecal sample. Sample

size was determined according to Thrusfield (1995) considering the recommendation for sample size estimation involving three or more cluster stages (Thrusfield, 1995). Based on this estimation, the estimated sample size was 1,152. Hence, a total of 1,147 cattle were tested, although 5% were not available for the reading after 72 h of tuberculin injection, and hence were considered as drop-outs. Thus, the final analysis of the data was based on the results of 1087 cattle tested.

Comparative intradermal tuberculin skin test (CIDT)

CIDT was carried out by injecting both bovine purified protein derivative (PPD) and avian PPD (Observe™ bovine and avian tuberculin, AsureQuality Company, Mt. Wellington, Auckland, New Zealand). Two sites on the skin of the mid-neck of the cattle, 12 cm apart, were shaved, and skin thickness was measured with a caliper. One site was injected with an aliquot of 0.1 ml of 2,500 IU/ml bovine PPD into the dermis, and the other was similarly injected with 0.1ml of 2,500 IU/ml avian PPD. After 72 h, the skin thickness at the injection sites was measured and recorded. Results were interpreted according to the recommendations of the Office International des Epizooties (OIE, 2009) at ≥ 4 mm cut-off and also at ≥ 2 mm cut-off (Ameni et al., 2008). Thus, at cut-off ≥ 4 mm, if the increase in skin thickness at the injection site for bovine PPD (PPD-B) was greater than the increase in skin thickness at the injection site for avian PPD (PPD-A) and PPD-B minus PPD-A was less than 2 mm, between 2 and 4 mm, or 4 mm and above, the animal was classified as negative, doubtful, or positive for BTB, respectively. At cut-off ≥ 2 mm, if the difference between B and A was greater or equal to 2mm, the animal was considered as positive, while if the difference is less than 2 mm, the animal was considered as negative. When the change in skin thickness was greater at PPD-A injection site, the animal was considered positive for mycobacterial species other than *Mycobacterium tuberculosis* complex. A herd was considered as positive if it had at least one tuberculin reactor animal.

Body condition scoring

The body condition of each of the study animal was scored using the guidelines established by Nicholson and Butterworth (1986). Accordingly, on the basis of observation of anatomical parts such as vertebral column, ribs, and spines, the study animals were classified as lean (score, 1 to 2), medium (3 to 4), or fat (greater than 5).

Fecal sample examination

Fecal samples from CIDT tested cattle were collected during tuberculin injection directly from the rectum of each animal using sterile glove and placed in labeled vials containing 10% formalin solution and then transported to the laboratory for microscopic examination using floatation technique (Soulsby, 1982), and eggs of the parasites were classified based on their morphology and size.

Data management and analysis

Data were classified, filtered, coded using Epidata software and Microsoft Excel sheet, and was transferred and analyzed using STATA version 11 (Stata Corp., Collage station, TX). Pearson chi-square was used to evaluate the statistical significance of the associations of different categorical variables with skin test results and McNemar's chi-square was used to assess the association of PPD-A and PPD-B results. Bivariate and multivariable logistic regression analyses were performed to quantify crude and adjusted effects of pre-specified risk factors on tuberculin reactivity. P-value less than 5% was considered statistically significant. In cases of estimating the effect of different risk factors in terms of odds ratio (OR) with corresponding 95% confidence interval, statistical significance was assumed if the confidence interval did not include one among its values.

RESULTS

Individual animal prevalence

On the basis of CIDT, the animal prevalence of BTB was 11% (119/1087) with 4 mm cut-off point and 18.4% (200/1087) with 2 mm cut-off point. At 4 mm cut-off point, there were statistically significant differences in proportions of bovine positive reactor animals between the four districts (χ^2 = 21.7, P = 0.000), herd size category (χ^2 = 8.72, P = 0.013), sex (χ^2 = 6.96, P = 0.008), age category (χ^2 = 21.12, P = 0.000) (Table 1). At 2 mm cut-off point, in addition to the factors indicted above, there was a statistically significant difference in proportion of bovine positive reactors between the pastoral and agro-pastoral production system (χ^2 = 3.8, P = 0.05) where a higher proportion of positive reactors in cattle under pastoral production system than those in agro-pastoral production system. Multivariable logistic regression analysis (Table 2) showed that older cattle (9 years and above) had 2.66 times the odds of being tuberculin reactors compared with those cattle less than 2 years old (adjusted OR = 2.66; CI =1.21-5.84). Cattle found in Amibara district had also the higher odds of being tuberculin positivity in relative to those cattle in Chifra district (adjusted OR = 6.56; CI = 1.63 to 28.73). At both cut-off points, there was no statistical significance difference in the proportion of bovine tuberculin positivity between groups in relation to body condition score, breed, gastrointestinal parasite infestation status, lactation status, reproductive status, and number of parity. The gastrointestinal parasite infestation status in general was low both in tuberculin nonreactors and reactor cattle. In majority of the tested animals eggs of *Trichostronglus* species were the most common parasite eggs identified in this study.

Herd prevalence

The herd prevalence was 44% (95% CI = 36 to 51%) and 56% (95% CI = 48 to 63%) at ≥ 4 mm and ≥ 2 mm cut-off points, respectively. In multivariable logistic regression analysis, herds found in Amibara district had the higher

Table 1. Association of different risk factors to skin test positivity at 4 mm cut-off point for bovine tuberculosis in Afar Pastoral Region of Ethiopia.

Variable	Number of cattle examined	Number of positive (%)	χ^2	p-value
Districts				
Chifra	106	2 (1.9)	21.768	0.000
Dubti	151	10 (6.6)		
Afambo	137	9 (6.6)		
Amibara	693	98 (14.1)		
Herd size				
<11	330	50 (15.2)	8.720	0.013
11≤X<31	533	50 (9.4)		
≥31	224	19 (8.5)		
Sex				
Male	112	4(3.6)	6.968	0.008
Female	975	115(11.8)		
Age*				
<2	183	9(4.9)	21.123	0.000
2-5	220	13(5.9)		
5-9	419	55(13.1)		
>9	265	42(15.9)		
BCS				
Poor	298	31(10.4)	0.140	0.932
Good	596	66(11.1)		
Fat	193	22(11.4)		
Production system				
Pastoral	937	109 (11.6)	3.271	0.071
Agro-pastoral	150	12 (6.7)		
GIT Parasite				
Absence	246	16(6.5)	2.811	0.094
Present	131	15(11.5)		
Lactation status				
Lactating	274	23(8.4)	2.453	0.117
Non-lactating l	277	14(5.1)		
Reproductive status				
Pregnant	174	12(6.9)	0.280	0.597
Non-pregnant	299	17(5.7)		
Parity (Calving) number				
<2	83	3(3.6)	3.776	0.151
2≤X<5	87	7(8.1)		
X≥5	47	6(12.8)		

*A given age range includes its lower bound and excludes its upper bound. BCS: Body condition score; GIT: gastrointestinal tract.

Table 2. Multivariable logistic regression analysis of tuberculin reactors with various host-related risk factors at 4 mm cut-off point.

Variable	Number of cattle examined	Number of positive in CIDT	Crude odds ratio (95% CI)	Adjusted odds ratio (95% CI)
Districts				
Chifra	106	2	1	1
Dubti	151	10	3.68 (0.79-17.18)	2.79 (0.58-13.45)
Afambo	137	9	3.65 (0.77-17.29)	2.49 (0.16-37.49)
Amibara	693	98	8.56 (2.07-35.27)	6.84 (1.63-28.73)
Herd size				
<11	330	50	1	1
11≤X<31	533	50	0.57 (0.38-0.88)	0.54 (0.35-0.85)
≥31	224	19	0.51 (0.29-0.90)	0.42 (0.23-0.77)
Sex				
Male	112	4	1	1
Female	975	115	3.61 (1.30-9.98)	1.66 (0.55-4.98)
Age*				
<2	183	9	1	1
2-5	220	13	1.21 (0.50-2.90)	1.05 (0.42-2.56)
5-9	419	55	2.92 (1.41-6.04)	2.11 (0.98-4.56)
>9	265	42	3.64 (1.72-7.68)	2.66 (1.21-5.84)
BCS				
Poor	298	31	1	1
Good	596	66	1.07 (0.68-1.68)	1.06 (0.66-1.70)
Fat	193	22	1.10 (0.62-1.97)	1.17 (0.62-2.18)
Production system				
Pastoral	937	109	1	1
Agro-pastoral	150	12	0.54 (0.27-1.06)	1.45 (0.15-13.41)

CI: Confidence interval; BCS: body condition scoring. *A given age range includes its lower bound and excludes its upper bound.

odds of showing tuberculin positivity in relation to those cattle in Chifra district (adjusted OR = 8.15; 95% CI = 1.77 to 37.59), and no significant association was found between herd positivity, herd size and production system (Table 3).

Association of tuberculin reaction to bovine and avian PPD

Comparative result of skin reaction to PPD-A and PPD-B is summarized in Table 4. Based on the ≥ 4 mm cut-off point, a statistically significant association was observed between the skin reaction to PPD-A (avian) and PPD-B (bovine) (χ^2= 75.98; p-value = 0.000). As indicated in Table 4, 0.5% of the tested cattle responded positively to both PPD-A and PPD-B. On the other hand, 10.5% of them reacted only to PPD-B, while 1.8% reacted only to PPD-A.

DISCUSSION

BTB is known to be endemic in Ethiopia (Hailemariam, 1975), and in spite of a good deal of studies carried out in Ethiopia in the last decade, very few addressed the

Table 3. Multivariable logistic regression analysis of herd positivity with selected herd risk factors at 4 mm cut-off point.

Variable	Number of herds examined	Number of positive herds (%)	Crude odds ratio (95% CI)	Adjusted odds ratio (95% CI)
District				
Chifra	17	2 (11.8)	1	1
Dubti	32	8 (25)	2.5 (0.47-13.39)	2.39 (0.44-12.93)
Afambo	13	6 (46.2)	6.42 (1.03-40.26)	5.71 (0.89-36.31)
Amibara	109	59 (54.1)	8.85 (1.93-40.58)	8.15 (1.77-37.59)
Herd size				
<11	99	39 (39.4)	1	1
11≤X<31	57	26 (45.6)	1.29 (0.67-2.49)	1.23 (0.61-2.48)
≥31	15	10 (66.7)	3.08 (0.98-9.69)	2.44 (0.75-7.94)
Production system				
Pastoral	158	87 (55.1)	1	1
Agro-pastoral	13	8 (61.5)	1.11 (0.36-3.44)	-

CI: Confidence interval; BCS: body condition scoring.

Table 4. Response of PPD-A and PPD-B* at 4 mm cut-off point.

PPD A result	Number (%) of animals with PPD-B result		Total number (%)
	Positive	Negative	
Positive	5 (0.46)	15 (1.4)	20 (1.84)
Negative	114 (10.48)	953 (87.67)	1067 (98.16)
Total	119 (10.94)	968 (89.05)	1087 (100)

*Positive and negative reactions were according to OIE guideline with skin indurations ≥4 mm and <4 mm, respectively, McNemar's chi-square=75.98; p-value=0.000.

epidemiology of BTB in pastoral cattle of the country, which owns 42% of the country's cattle population and occupied 61% of the landmass of the country (PFE et al., 2010). In the present study, a moderately high animal prevalence was recorded at ≥ 4 mm cut-off point. The highest was reported in Amibara (14.1%) and lowest in Chifra district (1.9%), indicating a variation in prevalence within the region. The overall prevalence obtained, in general, was higher than the previous reports from other pastoral area of Ethiopia and Uganda. Thus, in Ethiopia, from Hamer and Borna, 0.8 and 5.5% prevalence were reported by Tschopp et al. (2010) and Gumi et al. (2011), respectively, while in Uganda a prevalence of 1.3% was reported by Inangolet et al. (2008). It was also higher than the 4.1% prevalence in cattle under traditional extensive grazing system of Boji [western Ethiopia] which were reported by Laval and Ameni (2004). The difference might be related to the epidemiological factors that favors the transmission of BTB in the Afar Region, which include large herd sizes, communal grazing and watering of diverse species of animals including camel, cattle, goat and sheep, and an extensive seasonal mobility within and outside the districts, which creates favorable condition for wide range of interspecies contact (cattle, camel, goat, sheep and some wild animals such as Oryx, antelope and warthog).

In addition, the herds owned by individual pastoralist congregate together, forming a larger herd of the village and this larger herd composed of 500 to 600 of cattle and moves together to grazing and watering site. Such type of herd structure was observed particularly in Amibara district where conflict between Afar and Issa Somali tribes has been common and this intermixing of the herds might have increased the chance of contracting the infection, as it is demonstrated by higher prevalence in the district (14.1%) as compared to the others.

Moreover, during months of November to February, large number of livestock congregate in the cotton irrigation farms (2 to 4 weeks) to graze on the leftover of the cotton farm and different species of livestock (camel, cattle, and small ruminants) coming from neighboring zones and districts interact at the specific point, which create a favorable condition for close contact between animals and potential risk for transmission of diseases such as BTB among the animals. Such epidemiologically conducive conditions could lead to higher prevalence of BTB in the Afar Region as compared to the prevalence in other pastoral regions in Ethiopia. In addition, because of the presence of extensive range land in the districts, particularly in Amibara district, wild animals including oryx, gazelle, warthog and zebras (in and around Awash National Park) were observed grazing in close proximity with cattle which suggests a possible exposure for potential risk of disease transmission either way. The possibility of transmission of *M. bovis* between wildlife and cattle has been reported from other part of Africa and Europe (Woodford, 1982; Phillips et al., 2003; Cleaveland et al., 2005).

On the other hand, the result of the present study was much lower than the higher prevalence of BTB reported in urban intensive dairy farms of Ethiopia, where Holstein and crossbreeds cattle predominantly form the composition of the farms under intensive management system (Ameni et al., 2003, 2007). This difference might be mainly related to the intensive husbandry system practiced and the breed susceptibility (Ameni et al., 2007; Tsegaye et al., 2010). In our study, the animals tested were zebu Afar breed of cattle managed under extensive pastoral husbandry system which might be one reason for the differences in result, as the zebu breeds are known to be relatively resistant to BTB as compared to Holstein and other cross breeds managed under intensive system (Ameni et al., 2007,Cadmus et al., 2010).

The prevalence of BTB showed an increase with age and this finding was in agreement with previous reports by others (Kazwala et al., 2001; Oloya et al., 2006; Ameni et al., 2007; Inangolet et al., 2008; Regassa et al., 2010, Cadmus et al., 2010; Biffa et al., 2011). As indicated by these authors, the possible reasons could be the fact that older animals had longer and repeated chance of exposure to mycobacterial infection during their life time.

Furthermore, it has been observed that cows were more positive reactor than bulls, which is in agreement with other studies (Inangolet et al., 2008; Cadmus et al., 2010). In the Afar pastoral system, the majority (90%) of their herds is composed of cows kept exclusively for milk production and kept for longer time than the bulls, which form less than 10% of the herd, as bulls are sold or slaughtered in their early age. This condition might be the reason for higher tuberculin positivity in cows than bulls. As milk is consumed raw in Afar communities, the high prevalence in cows might create a potential risk for public health in the pastoralist community and need further investigation to identify its zoonotic significance and further design a control strategy in the region.

Similar to other studies in Ethiopia (Ameni et al., 2007; Tschopp et al., 2010; Gumi et al., 2011; Biffa et al., 2011), there was no association between body condition score and tuberculin skin test positivity. In addition, no statistical significant association was observed between tuberculin positivity and gastrointestinal parasite infestation which was different from previous finding by Ameni and Medihn (2000), which could be due to the difference in geographic locations and climatic condition which determines the existence and load of parasite in the area. In the present study with arid and semi arid climatic condition, the parasite infestation load was low, and immune compromising parasite such as *Fasciola hepatica* (Flynn et al., 2007, 2009) were not abundant as that of the highland area and hence might not have affected the overall tuberculin reactivity of the animals, while Ameni and Medhin (2000) did their study in highland where fasciolosis was highly prevailing.

Finally, because of the poor infrastructure facilities such as road accessibility and insecurity to the remote sites in the pastoral setting, part of the study was carried out with some level of convenient sampling method which can be taken as the limitation of this study.

Conclusion

To the best of our knowledge, this is the first BTB study done in Afar Pastoral Region of Ethiopia. The study revealed a moderately high prevalence of bovine tuberculosis in cattle and the presence of epidemiological risk factors for infection and transmission among cattle of the Region. Considering the fact that the Afar pastoral communities have very close contact with their animals and depend entirely on their livestock for subsistence through consumption of raw milk and other animal products, the findings of this study emphasizes the need for further investigation on isolation of the specific *Mycobacterium* species causing BTB in livestock, and their zoonotic significance in the Afar pastoral community, in order to design control options of the disease both in livestock and humans living in pastoral setting.

Abbreviations

BTB, Bovine tuberculosis; **CIDT,** comparative intradermal tuberculin skin test; **PPD,** purified protein derivative.

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